University of Alberta

ANTIBIOTIC RESISTANCE AND VIRULENCE OF *Campylobacter jejuni*

by

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Abstract

This thesis focuses on the characterization of *C. jejuni* isolates obtained from Alberta poultry between 1999 and 2001. Following characterization of antimicrobial-susceptibility profiles to 10 different antibiotics, resistance mechanisms were characterized. The isolates were also examined for the plasmid associated with virulence (pVir). A subgroup of *C. jejuni* isolates was further characterized for the presence of other virulence-associated genes and used in invasion assays of a Caco-2 cell model.

A high frequency of tetracycline-resistant *C. jejuni* were found in the isolates obtained from Alberta poultry. The presence of a plasmid carrying the *tet(O)* gene was found in 93% of the tetracycline-resistant isolates. The pVir plasmid was found to be carried in 63% of the *C. jejuni* isolates through DNA-DNA hybridization using two different sets of probes. Invasion assays using the Caco-2 cell model suggest that it is an appropriate *in vitro* model for detecting *C. jejuni* isolates that cause more severe gastroenteritis in humans.

An independent study into the mechanism of erythromycin resistance was also completed. The presence of a mutation in the three copies of the 23S rDNA, mutations in the ribosomal protiens L22 and L4 and the role of an efflux pump was analyzed in 23 isolates. For a majority of the isolates, a mutation in the 23S rDNA at position 2059 which results in a change from an A→G was found to be responsible for conferring macrolide resistance.

Several related research topics concerning *C. jejuni* are discussed in this thesis contributing to a further understanding of the organism.

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Table of Abbreviations

AFLP	Amplified fragment length polymorphism
APH	Aminoglycoside phosphotransferase
APLPH	Alberta Provincial Laboratory for Public Health
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
bp	Base pairs
BHI	Brain heart infusion
CadF	Campylobacter adhesion to fibronectin protein
CAT	Chloramphenicol acetyltransferase
CCDA	Charcoal cefoperazone deoxycholate agar
CDT	Cytolethal distending toxin
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CTP	Cytosine triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EF	Elongation factor
Ery	Erythromycin
FBS	Fetal bovine serum
GBS	Guillain Barré syndrome
GTP	Guanosine triphosphate
IPTG	Isopropylthiogalactoside
kb	Kilobase
LB	Luria-Bertani
LOS	Lipooligosaccharide

LPS	Lipopolysaccharide
MIC	Minimum Inhibitory Concentration
MH	Mueller Hinton
MLST	Multilocus sequence typing
MRPs	Macrorestriction profiles
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
QRDR	Quinolone resistance determining-region
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RND	Resistance nodulation divison
RPP	Ribosomal protection protein
rRNA	Ribosomal RNA
SEM	Standard error of mean
SDS	Sodium dodecyl sulphate
Str	Streptomycin
Tc	Tetracycline
Tfb	Transfer buffer
T4SS	Type IV secretion system
TTP	Thymidine triphosphate
UV	Ultraviolet
VBNC	Viable but non-cultureable
YE	Yeast Extract

Chapter 1

Introduction

1.1 An introduction to *Campylobacter jejuni*

Campylobacter jejuni is a foodborne and waterborne pathogen recognized as a major source of gastroenteritis worldwide (36). *C. jejuni* may contaminate milk, water, pork, beef, lamb, seafood, and poultry (108). Although human *C. jejuni* infections are usually self-limiting, the organism can cause severe complications including Guillain Barré syndrome (GBS) and Reiter's syndrome (13). The following is a discussion outlining what is known about *Campylobacter jejuni*, how it is transmitted through the food chain, and how it causes disease in humans. Furthermore the mechanisms of antibiotic resistance and the role which plasmids play in the ability of the organism to cause disease and resist the effect of tetracycline are reviewed.

1.2 The organism

Campylobacter jejuni is a gram-negative, spiral-shaped bacterium. The bacteria are usually motile by means of a single polar, unsheathed flagellum at one or both ends of the cell. The microorganism is microaerophilic, requiring oxygen and carbon dioxide concentrations of 3-5% and 3-15%, respectively (144). *C. jejuni* is frequently cultured at a temperature of 42°C to select it from other intestinal flora. *C. jejuni* typically requires a rich growth medium. The genome of *C. jejuni* is smaller than that of many bacteria of which the genomes have been sequenced, containing only 1.7-1.8 Mbp, and is A-T rich (GC content $\sim$ 30%) (23). Extra-chromosomal elements have been reported in *C. jejuni* (133). Although *C.*

jejuni is thought to have been observed as early as 1886, it was not until the 1970's with the development of improved isolation methods, and selective media, that *C. jejuni* was recognized as a major cause of bacterial gastroenteritis (33, 145).

1.3 Sources of *Campylobacter jejuni* in human infection

Human *C. jejuni* infections usually result from the ingestion of contaminated food (36). Common sources of *C. jejuni* are raw or undercooked poultry, unpasteurized milk, or untreated water (156). Close contact with pets, and activities in recreational water, are also possible sources of infection (16). Sporadic outbreaks of *C. jejuni* are usually the result of ingestion of poultry, whereas widespread outbreaks are usually due to ingestion of contaminated milk or water (23).

Within the animal host, *C. jejuni* is found to live as a commensal organism (144). The primary source of *C. jejuni* infection in humans is through consumption or contact with raw or under-cooked poultry (108). Studies have shown that chicks are rapidly colonized by *C. jejuni* within weeks of birth (108). There is no detectable colonization until the chicks are 10 days of age. Possible sources of *C. jejuni* colonization of poultry include feed and litter, water, humans, wild animals, and domestic animals (102). High population levels of *C. jejuni* (10^5 to 10^9 colony forming units per a gram) are present in the ceca of chickens (18). Birds carry *C. jejuni* throughout their life and as a result, the bacterium can easily be transmitted to humans through slaughter or food preparation of colonized birds. Eggs produced by poultry carrying *C. jejuni*, have not been associated with the spread of *C. jejuni* to humans, as the eggshell does not provide a suitable growth environment for the organism (65).

Wild birds are considered to be a reservoir for *C. jejuni* in the environment. One study in Sweden looked at the prevalence of *C. jejuni* in black-headed gulls. Strains of *C. jejuni* were isolated from humans, chickens, and the blackhead gulls and their DNA analyzed for similar macrorestriction profiles (MRPs) (19). Although the gulls were colonized by *C. jejuni*, the majority of isolates found differed in the genotype from those found in broiler chickens and humans. This suggests that the gulls, are only an accidental vehicle for human *C. jejuni* infection (19).

Other domestic farm animals associated with *C. jejuni* include pigs and cattle.

Pigs are considered to provide reservoirs for *Campylobacter* spp.; however, they tend to carry *C. coli*, which is seen less frequently in human infections. Unpasteurized milk from dairy cows was a major cause of *C. jejuni* outbreaks in the 1980's (41). Pasteurization is sufficient to eliminate *C. jejuni* from milk. Other dairy products including cheese and yogurt have not been found to harbour *C. jejuni* as the time required to ripen and the presence of lactic acid makes it difficult for bacteria to survive (29, 8).

Contaminated surface water is also recognized as a potential source of *C. jejuni* outbreaks. Cases of human *C. jejuni* infections could possibly occur due to accidental ingestion of recreational water (68). A recent example of contaminated water being responsible for an outbreak of diarrheal disease in humans occurred in 2000 in Walkerton, Ontario, Canada (26). Over 2300 people became ill from the consumption of water contaminated with fecal run-off from nearby farmland (142). Although the major organism identified in patients suffering from gastroenteritis was *Escherichia coli* O157:H7 (isolated from 13% of the patient stool samples), *C. jejuni* was also reported in 8% of the stool samples (142). This finding suggests that *C. jejuni* can exist and survive in the water supply, making water a possible source of *C. jejuni* outbreaks of gastroenteritis (67). Chlorination and proper treatment of the water is sufficient to kill *C. jejuni* (95) present in the water systems, which is suspected to originate from fecal contamination from domestic animals and wild birds (68).

Household pets are another source of infection, as kittens and puppies have been found to be carriers of *C. jejuni* and *C. upsaliensis* (120, 57). The importance of the role household pets play in the transmission of *C. jejuni* to humans is unclear.

1.4 Detection of *C. jejuni* in foods and survival in the environment

When a food-borne case or outbreak of *C. jejuni* does occur, it is usually very difficult to trace back to the source of infection. This problem arises due to the discrepancy between the time it takes to identify the source, and the ability of the organism to survive in the particular food over that time period. *C. jejuni* is very sensitive to environmental stresses, and a change in factors such as temperature and pH can easily kill the organism (145).

According to the Food and Drug Administration, a 25 gram sample of the suspected food is required. Different methods are used for preparing potential food sources for examination. With poultry, the carcasses are usually rinsed with peptone water. When water is the suspected source, 2-4 L of water is filtered and the filter is then enriched. With raw milk, the pH is adjusted to greater than 7.6 to inactivate the enzyme lactoperoxidase as it is toxic to *C. jejuni*. Once isolated, the organism is placed in a sufficient supplement and microscopic and biochemical tests are used in identification (38).

As *C. jejuni* is associated with foodborne disease, the bacteria must have some way to survive in the environment. It is believed that *C. jejuni* has a viable but non-culturable (VBNC) state (39). The existence of the VBNC state remains controversial, as the switch from the non-culturable form to the culturable form has not been observed (39). It is still unclear how the organism survives for long periods of time at cold temperature. Although it is known from sequence analysis that no genes encode for cold shock proteins, the VBNC form of the bacterium is one possible explanation for the ability of *C. jejuni* to survive at various temperatures (61).

1.5 Human infection

C. jejuni colonizes the human small intestine and colon. It is estimated that there are 2.4 million *C. jejuni*-related cases of gastroenteritis in the United States each year (43). In Canada, *C. jejuni* cause 12 000 cases of enteritis each year; however, there are likely many cases that go undocumented (M. Keelan, personal communication). It has been experimentally determined that an infective dose of 500 organisms is sufficient to cause disease in humans (15). The infective dose may vary with the susceptibility of the host and the virulence of the *C. jejuni* strain (15). In developed countries, the majority of *C. jejuni* infections result in a mild, watery diarrhea. Such cases require only that fluids and electrolytes be replenished and are typically self-limiting (2). *C. jejuni* infection can be associated with a number of other symptoms in addition to diarrhea and these may include: fever, intestine/abdominal pain, nausea, vomiting, headache, and the presence of mucous and blood in the feces (17, 124).

More serious complications of *C. jejuni* infection include septicemia, septic abortion, cholecystitis, and pancreatitis (16). Reiter's syndrome, better known as reactive arthritis, can sometimes follow *C. jejuni* infections (124). *C. jejuni*

has also been linked to the development of Guillain-Barré syndrome (GBS), a neurological disorder that results in flaccid paralysis (98).

GBS is an uncommon complication of *Campylobacter* infections occurring in approximately 1 in every 1000 cases (1). It has been observed that GBS follows infection with only certain serostrains of *Campylobacter*, in particular O19 strains (157). GBS is believed to be caused by an autoimmune reaction in which the body's immune system produces antibodies against gangliosides, which are part of the peripheral nervous system. Infection with a *C. jejuni* strain which bears ganglioside-like carbohydrate structures on the core oligosaccharides of their lipopolysaccharide (LPS [i.e. LPS which mimic the structure of gangliosides]) are thought to be responsible for generation of the antiganglioside antibodies. These antibodies are unable to distinguish between the LPS and the human ganglioside structure, leading to the pathological autoimmune response (6, 158).

1.5.1 Identification of *C. jejuni* infection

Campylobacter is isolated in clinical laboratories by plating stool specimens or filtrate on selective charcoal cefoperazone deoxycholate agar (CCDA) (74). It is routine practice in clinical laboratories to report isolates as *Campylobacter* spp. (55). Reference labs, at a later point in time, speciate *Campylobacter* isolates as the infection is considered a notifiable disease in Canada. Currently, the biochemical hippurate hydrolysis method is used for the identification of *C. jejuni* but this method is not always reliable (20, 34). Hippurate-negative *C. jejuni* strains have been found to exist and the test is therefore subject to interpretation (40, 118). With the sequencing of the genome of *C. jejuni*, PCR methods have also been developed in an effort to find easy, reliable, and reproducible tests to differentiate *C. jejuni* and *C. coli* from one another, as well as from other *Campylobacter* species (14, 20, 34, 76, 86, 104, 119, 128, 148). These PCR methods focus on the amplification of a gene or several genes (multiplex PCR) to distinguish each species. PCR methods which involve the amplification of a single gene often require yet another method to confirm the result or another PCR to identify another possible species. The possibilities of a specific and cost-effective PCR identification method are still being explored.

1.5.2 Typing

With *C. jejuni* being one of the most common causes of human bacterial gastroenteritis in the world (21), it would be most useful to study the epidemiology of infection. This would allow the design and implementation of effective interventions. Serotyping with heat labile antigens (Lior system), O serotyping based on lipopolysaccharide antigens (Penner system), biotyping, and phage typing are all phenotypic techniques that have been developed in efforts to differentiate *Campylobacter* strains (153). Molecular methods such as genotyping are considered to be more useful, as they can evaluate the phylogenetic as well as the epidemiological relationships among strains (153). Several methods including flagellin gene restriction fragment length polymorphism analysis (*fla*-RFLP), pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) are just a few examples of the technologies that allow for such evaluation (105).

MLST is a high resolution bacterial genotyping technique based upon the sequencing of gene fragments from a number of different housekeeping loci (127). This technique is advantageous and applicable for typing *Campylobacter* strains, as it is portable and highly reproducible (91).

PFGE is considered to be the most discriminatory method for typing *C. jejuni* and *C. coli* strains (103). Enzymes that cut the genome into a small number of fragments, such as *Sma*I, *Sal*I, and *Kpn*I, are typically used to digest the DNA in the PFGE process (23). The discriminatory power of this method makes it suitable for epidemiological investigations. PFGE has been used to group *C. jejuni* isolates from human outbreaks, allowing for the identification of genetically identical strains within the population (23, 37, 54). One criticism of this method is that it is fairly time consuming and costly, making it a less practical standard identification method.

The *fla*-RFLP is based upon the digestion of the*flaA* and/or *flaB* flagellin gene(s) product. These genes encode the proteins that make up the flagellar filaments of *C. jejuni*. These genes are highly conserved within individual isolates, but are very different between various isolates providing the basis of a typing scheme (103). A range of techniques and primer sets for the analysis of *flaA* have been developed in various laboratories making it difficult to compare the electrophoresis profiles obtained (3, 99, 107). The *fla* typing method is also considered to be less reproducible as there is evidence of recombination between the *flaA* and *flaB* genes of *C. jejuni* and the frequency of recombination is currently unknown (59). The search for a typing scheme for *Campylobacter* which is prac-

tical and discriminatory is still an on-going process.

1.5.3 Demographics

There are two sections of the population where *C. jejuni* infection occurs more frequently: children under four and young adults. The increased incidence in young children is believed to be due to greater reporting for this age group, as parents are more likely to seek medical care for their children when they become ill (43). The higher rate in young adults may be associated with a difference in the elements of their lifestyle, such as an increase in travel and consequent exposure to contaminated food or water (43). There is also a gender distribution, with *C. jejuni* infections occurring 1.2 to 1.5 times more often in males than in females (43, 123).

1.5.4 Treatment

With a severe or complicated infection, or when the patient is acutely ill (e.g. immunocompromised), antibiotic treatment is prescribed. Erythromycin has been the drug of choice, and continues to be used effectively in clinical treatment (100). Another popular drug used to treat *C. jejuni* infection is ciprofloxacin (100). Ciprofloxacin is an empiric prescription when the cause of diarrhea is unknown at the point of diagnosis (140). Though the antibiotics are still effective in a majority of cases, the emergence of quinolone-resistant strains of *C. jejuni* is a growing concern.

1.6 Virulence factors

Despite the fact that *C. jejuni* is a major food-borne pathogen, little is known about the precise mechanisms controlling pathogenesis. The structures and mechanisms involved in *C. jejuni* pathogenesis have been studied for the past thirty years and is still an area of active research.

Within the human host several virulence factors are necessary for *C. jejuni* to adhere to, colonize, and invade the intestinal epithelium (144). These factors include the flagella, invasion proteins, and the ability to produce toxins.

1.6.1 Flagellum: motility and chemotaxis

Motility is provided by the flagellum in combination with the cell shape. The flagellum helps the bacteria not only enter and traverse the mucus lining of the gut, but helps overcome the clearing movement of peristalsis (152). The flagellin subunits are encoded by the *flaA* and *flaB* genes which are known to undergo antigenic variation (60). The proteins (FlaA and FlaB) that make up the flagellum are a target of the human immune system, as demonstrated by a more pronounced immune response to these proteins in comparison to other proteins (152).

The bacterium must also have some method of effectively detecting that it is in an environment suitable for colonization, therefore, mucin (a component of mucus) is used as a chemoattractant (63). Takata *et al.* (131) demonstrated that chemotaxis was a key mechanism that ensured *C. jejuni* could colonize the gut through constructing non-chemotactic mutants, which were unable to colonize the intestinal tract of suckling mice. Once within its host, *C. jejuni* can either remain free-living in the mucous layer, or invade host epithelial cells (152).

1.6.2 Adherence

Enteric pathogens must adhere to the cells of the host gastrointestinal tract as the organism would otherwise be cleared by flowing fluids or peristalsis (69). The flagella as well as lipopolysaccharides on the outside of *C. jejuni* are thought to play a role in adherence (69). *C. jejuni* may produce fimbrial-like structures in the presence of bile salts, and that they may have some role in adhesion; but, this still remains to be confirmed (152).

Several genes, and the respective proteins which they encode, have been found to be important in the adherence of *C. jejuni* to intestinal cells. One of these proteins is the Campylobacter adhesion to fibronectin protein (CadF) which binds to fibronectin, a component of the extracellular matrix of the host cell (70). CadF is also involved in binding to host cell receptors which initiate a cell response leading to the internalization of the bacterium into the host cell (97). Four proteins (PEB1 thru 4), isolated from HeLa cells, may play an important role in the process of adhesion and invasion (111). Knockout mutants of *peb1* (encodes PEB1) reduces the ability of the *C. jejuni* strain to colonize and adhere to HeLa cells (111).

1.6.3 Invasion

Cellular invasion is an important pathogenic mechanism. This is supported by the observation that *in vivo* loss of cellular function and diarrhea occur (152). Kopecko *et al.* (72) have proposed that once *C. jejuni* has adhered to the apical surface of the intestinal epithelial cells, it secretes an effector protein (CiaB) into the host cell, triggering the extension of microtubules to form membrane protrusions around the bacterium. Internalization in an endocytic vacuole occurs and the *C. jejuni* is thought to exocytose to the baso-lateral surface of the intestinal epithelial cells. *C. jejuni* may then invade the neighbouring cells from the baso-lateral surface. The other possibility is for the *C. jejuni* to be taken up by lymphocytes of the host, which are attempting to clear the organism, but as they travel through the blood system to the lymph nodes, they may actually aid in the local dissemination of *C. jejuni* (72).

Invasion has been demonstrated in a number of cell lines including INT407, HEp2, and Caco-2 cells (152). There is much debate over which particular cell line should serve as an appropriate cell model. Most recently, there has been a tendency to use Caco-2 cells as they are considered to be the most representative of the *in vivo* cell type that *Campylobacter* would encounter (9, 22, 44, 58, 71, 80). Caco-2 cells are able to form well-differentiated monolayers, with tight junctions and brush border enzymes mimicking the conditions of the small intestinal epithelial cells (44).

Recently, the plasmid, pVir, was identified in a highly invasive *C. jejuni* strain 81-176 (9). The pVir plasmid is approximately 37.5 kb and contains components of a type IV secretion system (T4SS) (10). T4SS are macromolecular transfer systems used for the intercellular transfer of plasmids, or in bacterial pathogens, can also be used to transfer effector proteins into eukaryotic host cells (25). Seven genes of pVir have been revealed to encode orthologues of T4SS proteins (encoded by *vir* genes). Decreased ability of *C. jejuni* to successfully invade INT407 cells was found when certain *vir* genes were deleted. This suggested that the genes encoded on the plasmid are essential for successful invasion into host cells (10). Furthermore, the ability of a strain in which the a *virB11* homolog (*cjp5*) had been mutated, reduced the virulence of *C. jejuni* 81-176 in the ferret disease model also suggested that the plasmid does play a role in aiding invasion (10).

1.6.4 Toxin production

C. jejuni is also known to produce toxins, which aid in causing infection within the human gut. Though the genome of *C. jejuni* has been sequenced, few virulence factors were identified (109). The action of several cytotoxins has been studied in various strains (152). Of these, cytolethal distending toxin (CDT) is best understood and its genetic locus has been cloned and sequenced (80). CDT's cytotoxic effect is a result of its ability to block the host cell cycle in the G2 phase, likely due to inactivation of CDC2 kinase required for mitosis (155). CDT has been proposed to be the cause of diarrheal symptoms associated with *C. jejuni* infections but its actual role remains to be proven (144).

There have been many other reports of the various toxins produced by *C. jejuni* and their interactions with the various cell lines. In all cases, the toxin caused the cells to become round in shape and die (112). Each study utilized different methodologies and different strains. Unfortunately, the identification of the toxin-producing genes and/or the isolation of the toxins was unsuccessful (112).

C. jejuni also possesses lipopolysaccharides (LPS) on its surface. The lipid A portion of the LPS has endotoxic activity, and can lead to sepsis and shock when it is released from the surface of the bacteria in systemic infections (152). Some strains of *C. jejuni* may also produce lipooligosaccharides (LOS). The structure is quite diverse in strains of *C. jejuni* and may contain N-acetylneuraminic acid, which is more commonly found in vertebrate glycoproteins and gangliosides and is rare in bacteria (152). Molecular mimicry may play a role in the development of GBS (56).

1.7 Antimicrobial resistance

Antibiotics are used to kill or inhibit the growth of bacteria. They are small, low-molecular-weight compounds that usually have specific targets in the bacterial cell. They act to inhibit cell processes, which are vital to the organism's survival. Bacteria have been able to adapt to the use of antibiotics. Four main mechanisms of resistance to antibiotics have been observed, these are: modification or destruction of the antibiotic; protection of the antibiotic target site; efflux of the antibiotic from the host cell; and alteration of the antibiotic target site (31).

The incidence of antimicrobial resistance has increased for *C. jejuni*, but the reasons for the acquired mechanisms and the potential source is uncertain (36,

45, 52). Antibiotics are used in animal feeds to treat infection and as growth promoters by administering the drug at sub-therapeutic levels (93). Antibiotics are usually fed to farm animals through mass medication procedures also referred to as metaphylaxis (93). The purpose of such mass administration is to treat those animals that are sick, and prevent the disease from spreading to healthy animals (93). Typically the drugs are administered at therapeutic levels over short periods of time. When antibiotics are fed to healthy animals, the intestinal flora of the animals can provide a reservoir for antibiotic-resistant bacteria. Such feeding practices can pose a risk to human health, as antibiotic-resistant bacteria that are pathogenic to humans may be selected in the animals. During slaughter or food preparation, foodstuff can potentially be contaminated and cause infection (82). It is also possible that antibiotic resistance may be conferred upon non-pathogenic organisms in the animal host. In the human gut, resistance could be transferred to an organism that is pathogenic (82).

Within the clinical setting, an increase in resistance to the antibiotic tetracycline has been seen. In Quebec, Canada a recent study reported that 56% of *C. jejuni* isolates collected between 1995-97 were tetracycline-resistant, in comparison to the 19% reported for 1985-86 (45). In Alberta, analysis of 203 clinical isolates of *C. jejuni* showed a significant increase in the proportion of tetracycline-resistant isolates to 49.8% (1999-2002) from the 8.6% reported in 1981 (53). In both recent studies, the antimicrobial resistance to other antibiotics remained low. Tetracycline resistance is proposed to be widespread in clinical isolated due to the use of tetracycline in veterinary medicine (93). The *tet(O)* gene has been observed to rapidly and spontaneously transfer without antimicrobial selection pressure between *C. jejuni* strains in the digestive tracts of poultry (7).

Since the early 1990's, there has been an increase in the prevalence of *C. jejuni* isolates from humans and poultry that are resistant to fluoroquinolones. Studies compiled in the Netherlands were the first to report the emergence of fluoroquinolone resistance in human clinical isolates (35). The fluoroquinolone, enrofloxacin, was introduced in the Netherlands for treatment of infections in poultry. Following the introduction of this antibiotic, frequencies of ciprofloxacin resistance increase to 11% in humans and 14% in chickens as compared to a 0% resistance profile prior to its use (35).

Within Alberta erythromycin is the drug of choice for treating *Campylobacter* (53). Less than 1% of isolates were observed to be resistant to erythromycin (53). Within the following sections, the details of antibiotic resistance mechanisms *C.*

jejuni uses will be discussed briefly.

1.7.1 Tetracycline resistance

Tetracycline is a broad-spectrum antibiotic with bacteriostatic and reversible effects. The drug works by binding to the 30S subunit of the bacterial ribosome at a high-affinity site and several low-affinity sites. In doing so it prevents binding of aminoacyl-tRNA to the A site (140). Unlike many other gram negative bacteria where tetracycline resistance is mediated by efflux proteins, resistance in *C. jejuni* to tetracycline is due to Tet(O), an elongation factor like protein that is usually carried on a self-transmissible plasmid (140).

Tet(O) is a ribosomal protection protein that shares similarity with Tet(M), Tet(P), Tet(Q), Tet(S), Tet(T), Tet(W) and Otr(A) found in other species of bacteria (24). Tet(O) promotes the release of bound tetracycline by interacting with the 70S ribosome. Through chemical probing assays, Tet(O) has been demonstrated to change the reactivity of the 16S rRNA (28). Thus it has been proposed that Tet(O) does not interfere directly with tetracycline binding but acts allosterically (126). Connell *et al.* (28) have presented evidence that suggests Tet(O) and tetracycline induce conformational changes in the ribosome but in functionally opposite directions. The conformational change Tet(O) induces does not allow reversion to the original conformation once it disassociates from the ribosome (28).

Sequence similarity between the N terminal regions of Tet(O) and the ribosomal elongation factors EF-Tu and EF-G has been found (134). It has been hypothesized that Tet(O) may act as a molecular mimic of EF-G and EF-Tu possibly by binding to and causing a conformational change in the ribosome promoting the release of tetracycline (28). DNA probes have played a major role in identifying whether the *tet(O)* gene is present on chromosomal DNA or plasmids from *C. jejuni* (79).

1.7.2 Erythromycin resistance

Erythromycin is the drug of choice for the treatment of *C. jejuni* enteritis as it is safe and effective (36). The drug works by binding to the ribosome preventing the dissociation of the peptidyl tRNA (140). An increase in the number of *C. jejuni* strains resistant to erythromycin has been observed over the past 20 years possibly due to the use of antimicrobials in agriculture (5). Two point mutations

at positions 2074 and 2075 (2058 and 2059 positions using *E. coli* numbering) in the V domain of the 23S rRNA gene are responsible for bacterial resistance to erythromycin (143). In *C. jejuni*, mutations usually involve replacement of an adenine residue with a cytosine at position 2074 or a guanine residue at position 2075 (143). The emergence of erythomycin resistance in *C. jejuni* isolates is cause for concern as limited treatment options are currently available.

1.7.3 Fluoroquinolone resistance

Fluoroquinolones are chemically modified forms of the quinolone antibiotic nalidixic acid. Fluoroquinolones were once considered useful in treating human *C. jejuni* infections, as the organism was highly susceptible and high concentrations could be attained in the lumen without toxic effects to the individual (113). Ciprofloxacin was one of the most widely used antibiotics in the world and second choice for treating gastroenteritis in humans (140). These antibiotics target the type II topoisomerase and topoisomerase IV which are essential enzymes for DNA replication, recombination, and transcription to take place (140). Quinolones are able to inhibit the DNA gyrase by forming a ternary complex between the gyrase and DNA, which leads to cleavage of the DNA following complex formation (12).

A mutation in the quinolone resistance determining region (QRDR) of the *gyrA* gene, encoding the GyrA subunit of DNA gyrase, is responsible for resistance of *C. jejuni* to fluoroquinolones. A substitution at Thr 86, Asp 90, or Ala 70 in GyrA typically results in resistance (113, 150). The presence of Thr-86-Ile in GyrA is the most common mutation (113). Occasionally resistance to fluorquinolones has been found to be due to a mutation in *parC* (35).

Resistance of *Campylobacter* spp. to ciprofloxacin may also be due to the work of a multi-drug efflux pump, the *cmeABC* gene operon (81). Lin *et al.* (81) conducted experiments in which the disruption of the CmeABC efflux pump resulted in intracellular accumulation of ciprofloxacin as well as the various other antimicrobial agents. However, a recent study by Payot *et al.* (81) suggests the role of the efflux pump in resistance to fluoroquinolones is minor.

1.7.4 Resistance to other antibiotics

Campylobacter have acquired a chloramphenicol acetyltransferase (CAT). Chloramphenicol normally competes with the aminoacyl-tRNA for the A-site of the ribosome (140, 151). Modification of chloramphenicol by the CAT prevents the

antibiotic from binding to the ribosome (140, 151). This determinant was cloned and sequenced previously by Wang and Taylor (151).

Trimethoprim selectively inhibits the enzyme dihydrofolate reductase. *Campylobacter* has become resistant to this drug through the production of a trimethoprim-resistant dihydrofolate reductase. Gibreel and Skold (50) analyzed several strains with high levels of trimethoprim-resistance. The chromosomally-located determinants were cloned and found to mediate high levels of trimethoprim-resistance in *Escherichia coli*. The *dfr1* gene is most commonly found determinant of trimethoprim resistance in *C. jejuni*. At least 10% of the strains of *C. jejuni* examined by Gibreel and Skold (50) were found to have both *dfr1* and *dfr9* genes encoding distinct dyhydrofolate reductases. On either side of *dfr1*, it was observed that there were integron-like features and remnants of a transposon (Tn*5393*) surrounding *dfr9*. The *dfr9* gene surrounded by Tn*5393* has also been found to exist in *E. coli* isolates from humans and swine, suggesting that the acquisition of the *dfr* genes by *C. jejuni* resulted from high levels of antibiotics being fed to domestic animals (51).

Aminoglycosides, such as kanamycin, are bactericidal. They bind irreversibly to the ribosome and inhibit protein synthesis, but the actual killing mechanism has not been elucidated. Some strains of *Campylobacter* possess an enzyme referred to as an aminoglycoside phosphotransferase (APH) which modifies the antibiotic making it no longer effective (140). Determinants of aminoglycoside resistance have also been shown to be plasmid-mediated (53).

1.8 Concluding Remarks

Campylobacter jejuni has been recognized as a common cause of human gastroenteritis since the late 1970's. It has also become known as a major food-borne pathogen, with poultry being the main vehicle of transmission to humans. The characteristics of *C. jejuni* including its virulence factors, ability to acquire antibiotic resistance, sources of infection, and the disease symptoms it causes in humans have been discussed. There still remains a great deal to be discovered about *C. jejuni* and the mechanisms by which it causes disease in humans. *C. jejuni* is an important pathogen within the food chain and our water supplies. For the time being, public educations programs that inform the population on proper food handling and adherence to regulations and best practices by the food industry, will aid in preventing the transmission of *C. jejuni*. The problem of *C. jejuni* enteritis will

remain until further knowledge is acquired to develop better control measures.

This thesis focuses on the characterization of *C. jejuni* isolates obtained from Alberta poultry between 1999 and 2001. Following characterization of antimicrobial-susceptibility profiles to 10 different antibiotics, resistance mechanisms were characterized. The *tet(O)* gene which is associated with conferring tetracycline resistance, and mutations in the *gyrA* gene associated with ciprofloxacin resistance were investigated in further detail.

The isolates were also examined for the plasmid pVir, associated with virulence. A subgroup of *C. jejuni* isolates was further characterized for the presence of other virulence associated genes and used in invasion assays of a Caco-2 cell model.

An independent study into the mechanism of erythromycin resistance was also completed as part of the work presented in this thesis. The role of the 23S rDNA mutation, efflux pumps, and ribosomal proteins involved in mediating this resistance were further explored. Several related topics concerning *C. jejuni* are discussed in this thesis and contribute to a further understanding of the organism within the respective areas.

Chapter 2

Materials and Methods

2.1 *Campylobacter* isolates used in this thesis

2.1.1 Erythromycin-resistant *Campylobacter* isolates

This study was carried out using 23 macrolide-resistant isolates of *Campylobacter* (Table 2.1). All isolates were cultured on Brain Heart Infusion (BHI) agar (Difco, Beckton-Dickinson, Sparks, MA) supplemented with 0.4% yeast extract (YE) (Difco) and incubated at 37°C under microaerobic conditions (5% CO_2, 10% H_2, balance N_2) for 24-48 h. Stocks were stored at -80°C in 20% glycerol BHI-YE broth.

The isolates were identified as *Campylobacter* using the following tests: catalase production, hippurate hydrolysis, indoxyl acetate hydrolysis, H_2S production in triple sugar agar, and susceptibility to nalidixic acid and cephalothin (Table 2.2). To identify the *C. jejuni*, strains, the hippurate hydrolysis test was used on all of the isolates, and the 11 isolates from Quebec were also tested using the API Campy Kit (API bioMerieux SA, Marcy l'Etoile, France). The API Kit consists of ten enzymatic, six inhibition, and three susceptibility tests (64) (Table 2.3). To obtain pure cultures, subcultures were made by re-streaking single colonies onto fresh Mueller Hinton (MH, Difco, Beckton-Dickinson, Sparks, MA) plates which were then incubated under the same conditions as designated above.

2.1.2 *C. jejuni* isolates from poultry

The 104 *C. jejuni* isolated from poultry carcass washings were supplied by Mary VanderKop and John Girvan (Agri-Food Laboratories, Alberta Agriculture,

Table 2.1. Characteristics and identity of the *Campylobacter* isolates used in the study of erythromycin resistance.

Isolate	Source (Location)	Year of Isolation	Original Classification	Erythromycin MIC (μg/mL)
UA37	Clinical (Belgium)	1974	*C. coli*	>1024
UA29	Sheep (Guelph, Canada)	1980	*C. coli*	>1024
UA261	Clinical (Southampton, UK)	1982	*C. coli*	>1024
UA336	Cattle (Southampton, UK)	1982	*C. coli*	>1024
UA40	Clinical (Brussels)	1982	*C. coli*	256
UA609	Clinical (Ottawa, Canada)	1984	*C. coli*	>1024
UA585	Clinical (Wales)	1984	*C. coli*	>1024
UA749	Clinical (Belgium)	1989	*C. coli*	>1024
001A-15	Clinical (Quebec Canada)	2000	*C. coli*	>1024
001B-17	Poultry (Quebec, Canada)	2001	*C. coli*	>1024
001B-40	Poultry (Quebec, Canada)	2001	*C. coli*	>1024
001B-15	Poultry (Quebec, Canada)	2001	*C. coli*	128
UA709	Clinical (The Netherlands)	NA[a]	*C. jejuni*	>1024
UA 697	Clinical (United Kingdom)	1984	*C. jejuni*	512
UA695	Clinical (Edmonton, Canada)	1986	*C. jejuni*	>1024
UA710	Clinical (Ottawa, Canada)	1987	*C. jejuni*	>1024
001A-18	Clinical (Quebec, Canada)	2000	*C. jejuni*	256
001B-22	Poultry (Quebec, Canada)	2001	*C. jejuni*	256
001A-115	Clinical (Quebec, Canada)	2001	*C. jejuni*	512
001A-114	Clinical (Quebec, Canada)	2001	*C. jejuni*	512
001A-168	Clinical (Quebec, Canada)	2001	*C. jejuni*	512
001B-34	Poultry (Quebec, Canada)	2001	*C. jejuni*	512
001B-31	Poultry (Quebec, Canada)	2001	*C. jejuni*	512

[a]NA= Not available

Table 2.2. Original laboratory tests used to speciate *C. jejuni* and *C. coli*.

	Growth			[redacted]								Susceptibility	
Organism	42°C	1% Glycine	3.5% NaCl									Nalidixic acid	Cephalothin
C. jejuni	+	+	-	+	+	-	+	-	-	+	+	S	R
C. coli	+	+	-	+	+	-	+	-	-	+	-	S	R

+= growth, reaction
-= inhibited, no reaction
S= senstive
R= resistant

Table 2.3. Tests present in the API Campy ® system.

	Enzymatic																	Growth Inhibition		
	Urease	Reduction of Nitrates	Esterase	Hippurate	Gamma Glutamyl Transferase	Reduction of Triphenyl Tetrazolium Chloride	Pyrrolidonyl Arylamidase	L-Arginine Arylamidase	L-Aspartate Arylamidase	Alkaline Phosphatase	Production of H_2S							Nalidixic Acid	Sodium Cefazoline	Erythromycin
C. jejuni	-	+	+	+	+	+	V	-	-	+	-	-	+	-	V	-	-	-	+	-
C. coli	-	+	+	-	-	+	-	+	-	+	-	-	+	+	-	-	-	-	+	+

+ = Presence of growth or enzyme or resistance to substrate.
- = Inhibition of growth, absence of enzyme or susceptibility to substrate.
V = Results may vary between strains.

Food and Rural Development). Original isolation and identification of *C. jejuni* was performed by Alberta Agriculture. Identification was confirmed by PCR analysis of the hippurate gene (*hipO*, see section 2.5.1). Isolates were transported from Alberta Agriculture as swabs stabbed into transport medium stored at 4°C. Swabs were inoculated onto BHI-YE agar and incubated under microaerobic conditions for 24 h at 37°C.

2.2 Hippurate hydrolysis assay

A standardized hippurate hydrolysis test was performed using the procedure outlined previously (137). A loopful of bacteria from a 24 h plate was suspended in 0.4 mL of a 1% sodium hippurate solution and incubated in a 37°C water bath for 2 h. After the incubation period, 3.5% ninhydrin was added and the tubes were observed for a change in color. A positive hippurate reaction was indicated by the appearance of a deep purple color within 10 min. Each isolate was tested in duplicate with *C. jejuni* NCTC11168 as a positive control and *C. coli* UA585 as a negative control.

2.3 Antibiotic susceptibility testing and MICs

Antibiotic discs (Oxoid, Nepean, ON) were used in the disc-diffusion method to determine the susceptibility of *C. jejuni* to ten antibiotics: tetracycline (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), kanamycin (30 μg), imipenem (10 μg), doxycycline (30 μg), gentamicin (10 μg), and amoxicillin-clavulanic acid (30 μg). E-tests (AB BIODISK, Dalvägen, Sweden) were also completed to determine the minimum inhibitory concentrations (MICs) for the *C. jejuni* isolates to the ten antibiotics.

Suspensions of *C. jejuni* were made in BHI-YE broth (Difco-Becton-Dickinson, Sparks, MA) to the equivalent of a McFarland turbidity standard of 1.0 (OD_{625}= 0.22 ± 0.02) and spread in three directions, onto MH agar containing 5% defibrinated sheep blood (Dalynn, Calgary, AB) using a sterile swab that was dipped into the bacterial suspension. Antibiotic discs and E-test strips were applied using sterile forceps. The plates were read after incubation for 48 h at 37°C under microaerobic conditions generated using CampyGen Pacs (Oxoid, Nepean, ON). The zones of inhibition were measured on the plates containing Oxoid discs and determined to be resistant, intermediate, or susceptible based upon the guidelines outlined by

the Clinical and Laboratory Standards Institute (CLSI, previously NCCLS). With the E-test, the MIC was determined as the point where the end of growth distinctly intersected with the strip. When growth of the strain being tested was all the way along the strip, the MIC was reported as greater than the highest value on the scale of the strip.

Breakpoint criteria used for the various antibiotics are listed in Table 2.4. Recent internationally accepted criteria for susceptibility testing for *Campylobacter* species is now available for certain antibiotics which were tested (92), and the discrepancies between the methods suggested and those used in this thesis are discussed at a later point in this thesis (Section 4.1).

All isolates which were shown to be resistant to any antibiotic by either or both methods, or which showed any discrepancy in the level of resistance determined, were tested using the agar dilution method. Suspensions of the *C. jejuni* isolates were inoculated onto MH agar with 5% defibrinated sheep blood, containing different concentrations of the various antibiotics using a Steer's replicator. Plates were incubated for 48 h under microaerobic conditions at 37°C. The MIC was defined as the antibiotic concentration at which the bacterial growth was completely inhibited as recommended by the CLSI (same as E-test concentrations listed in Table 2.4).

Antibiotic-susceptibility testing was completed using three different methods for the 104 *C. jejuni* isolates obtained from Alberta Agriculture. For isolates used in the erythromycin study, determination of MICs of erythromycin (8 μg/mL), clarithromycin (8 μg/mL), and tetracycline (16 μg/mL) was performed using the agar dilution method, whereas determination of ciprofloxacin MICs (1 μg/mL) was carried out using the E-test.

Table 2.4. Breakpoint definitions for *C. jejuni* to various antibiotics using E-test, agar dilution, or disc diffusion.

Antibiotic	E-test and agar dilution MIC breakpoints (µg/mL)	Oxoid Disc Inhibition Zone R≤ "X"mm < I < "Y"mm≤ S[a]	
		X	Y
Amoxicillin:clavulanic acid (2:1)	32/16	13	18
Chloramphenicol	32	12	18
Ciprofloxacin	4	14	19
Doxycycline	16	12	16
Erythromycin	8	13	23
Gentamicin	16	12	15
Imipenem	16	13	16
Kanamycin	16	13	18
Nalidixic acid	32	13	19
Tetracycline	16	14	19

[a]R= resistant
I= intermediate
S= susceptible
Interpretation for disc diffusion are those values set for *Enterobacteriaceae* with the exception of erythromycin for which the interpretation criteria for *Staphylococcus* spp. is used.

2.4 Isolation of chromosomal and plasmid DNA

Chromosomal DNA was isolated from all *C. jejuni* strains, using a Wizard Genomic DNA purification Kit (Promega, Madison, WI). All chromosomal preparations were stored in the DNA rehydration buffer provided with the kit and stored at -20°C.

Plasmid DNA was obtained using Qiagen Spin Mini Kits and Qiagen Hi-Speed Midi Kits (Qiagen, Mississauga, ON). Plasmid preparations were eluted from the columns provided with the kits using sterile Milli-Q water (500 μL). The plasmid extracts were analyzed for the presence of the pTetO and pVir plasmids using PCR with primers specific for the genes associated with the plasmids (Tables 2.8 and 2.9). All total and plasmid DNA preparations were quantified using a spectrophotometer (Ultraspec 3000, Pharmacia Biotech, Piscataway, NJ).

2.5 PCR protocols

All PCR reactions were carried out in a Perkin-Elmer thermocycler (Perkin-Elmer, Norwalk, CT). All PCR reactions were carried out using Taq DNA polymerase (Invitrogen, Burlington, ON) unless otherwise indicated. PCR products were separated on 1% agarose gels (Gibco, Burlington, ON), stained with ethidium bromide, and visualized on a UV transilluminator (Fisher, Burlington, ON). Gel images were captured using Kodak Digital Imaging Software.

2.5.1 Amplification of *C. jejuni* and *C. coli* specific genes

PCR amplifications of *hipO*, *mapA* and *aspA*, were completed according to the methods reported by Burnett *et al.* (20) (Table 2.5). PCR-RFLP analysis of the highly variable region of the 23S rDNA PCR products was performed following digestion with *Alu*I and *Tsp*509I as outlined previously (40) (Table 2.5).

2.5.2 Multiplex-PCR conditions

Concurrent amplifications of the *hipO* gene and the *aspA* gene were performed in a total volume of 50 μL containing 0.2 μM of each of the *aspA* primers, and 0.8 μM of the *hipO* primers; 1x PCR Reaction Buffer [500 mM KCl, 200 mM Tris-HCl (pH 8.4)]; 200 μM of dATP, dTTP, dCTP, and dTTP; 2 mM $MgCl_2$; 300 ng of genomic DNA, and 1U of Taq DNA Polymerase (Invitrogen, Burlington,

ON). Thirty cycles of amplification consisted of a 1 min denaturation step at 94 °C, a 1 min annealing step at 58 °C, a 1.5 min extension at 72°C, and a final extension of 72°C for 10 min was carried out. Isolates previously determined to be either *C. jejuni* (NCTC11168, 81-176) or *C. coli* (UA585, 001A-18) were run as controls. A mixture of the genomic DNA preparations of *C. jejuni* and *C. coli* were also run as a control to ensure that correct amplification of both the *hipO* and *aspA* products occurred in the presence of a mixed culture. Several strains of other *Campylobacter* species including *C. fetus* subsp. *venerealis* (UA811, UA814), *C. fetus* subsp. *fetus* (UA553, UA781), *C. upsaliensis* (UA1080, UA1079), *C. hyointestinalsis* (UA565, UA566), *C. lari* (UA603, UA591), and *C. sputorum* (UA655) were also run to confirm that the primers were specific for the identification of *C. jejuni* and *C. coli*.

2.5.3 Determination of the mechanism of macrolide resistance

For the *C. coli* isolates, an internal part of domain V of the 23S ribosomal DNA (300 bp) was amplified by PCR using primers DP1 and CJ1 (Table 2.6) and Table 2.7). The PCR products were purified using a PCR purification kit (Qiagen, Mississauga, ON) and then the DNA sequenced. Different copies of the 23S rRNA gene of the *C. jejuni* isolates were amplified by PCR using three oligonucleotide pairs, FI-CJ copy-R, FII-CJ copy-R or FIII-CJ copy-R (Table 2.6 and Table 2.7). Amplification was performed in a total volume of 50 μL containing the PCR primers at 0.1 μM each; 1x AccuPrime PCR Buffer II [60 mM Tris-SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$, 0.4 mM Mg_2SO_4, 0.2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, thermo-stable AccuPrime^{TM} protein, 1% Glycerol]; 200 ng of the genomic DNA; and 1U of AccuPrime^{TM} Taq DNA Polymerase (Invitrogen, Burlington, ON). The PCR products were analyzed by gel electrophoresis, purified and sequenced using the primer DP1.

Genes coding for L4 and L22 ribosomal proteins were amplified by PCR using the PCR reaction mixture mentioned above using primers CJ18, CJ19, CJ20 and CJ21 (Table 2.7), purified and sequenced.

2.5.4 PCR conditions for identification of the *tet(O)* gene

PCR was used to amplify a 559 bp product within the *tet(O)* gene using primers DMT1 and DMT2 (Table 2.8). PCR conditions consisted of 1 min 95°C, 1 min 50°C, and 1 min 72°C. Templates used included plasmid and chromosomal

Table 2.5. Primers and PCR conditions used in the identification of 23 erythromycin-resistant *Campylobacter* isolates.

Primer Pair	Target	PCR conditions			Product Size (bp)	Reference
		denaturation	annealing	extension		
Hip 1 Hip 2	*hipO*	94 (60s)	66 (60s)	72 (60s), 25 cycles	735	(83)
CJAG48-1L CJAG48-1R	*mapA*	94 (60s)	43 (60s)	72 (60s), 35 cycles	641	(129)
CC18F CC519R	*aspA*	94(60s)	60 (60s)	72 (60s), 25 cycles	500	(83)
THERM1 THERM4	*23S rRNA*	94(60s)	56 (60s)	72 (60s), 35 cycles	491	(40)

Table 2.6. 23S rRNA primer sequences and binding location in the *C. jejuni* genome.

Primer	Sequence (5'→3')	Positions[a]
CJ1	TCAAGCTGGTTAGCTA	4805-4789
DP1	ACGGCGGCCGTAACTATA	4497-4515
CJ copy-R	CTACCCACCAGACATTGTCCCAC	4839-4818
FI	CCCTAAGTCAAGCCTTTCAATCC	38701-38723
FII	CGTTATAGATACGCTTAGCGGTTATG	393561-393587
FIII	CATCGAGCAAGAGTTTATGCAAGC	695881-695905
CJ18	GAAGTTGTATCTTATGATGCTGAAAA	134353-134328
CJ19	TAACTACGACACCTTGCTCTTG	133563-133584
CJ20	TCCGGTTTATATTACTGAA	132353-132335
CJ21	CTTTAGTTGGAAACCATCTTG	131731-131751

[a] Numbering used for the 23S rRNA gene of *C. jejuni* (accession no.Z29326) used for primers CJ1, DP1, and CJ copy-R. For the other primers the numbering is based on the complete genome sequence of *C. jejuni* (accession no. NC_002163).

Table 2.7. PCR primers and conditions used to determine the mechanism of macrolide resistance.

Primer Pair	Target Gene	No. of Cycles	PCR conditions					Product size
			Hot Start	Denaturation	Annealing	Extension	Final Extension	
CJ1 DP1	23S rDNA	30		95°C, 30 sec	52°C, 1 min	72°C, 1min		300 bp
FI-CJ[a]	23S rDNA Copy I	25	95°C, 2 min	95°C, 30 sec	55°C, 30 sec	68°C, 7 min	68°C, 15 min	5.7 kbp
FII-CJ[a]	23S rDNA Copy II	25	95°C, 2 min	95°C, 30 sec	55°C, 30 sec	68°C, 7 min	68°C, 15 min	5.8 kbp
FIII-CJ[a]	23S rDNA Copy III	25	95°C, 2 min	95°C, 30 sec	55°C, 30 sec	68°C, 7 min	68°C, 15 min	5.7 kbp
CJ18 CJ19	L4	30		94°C, 1 min	60°C, 1 min	72°C, 1 min		800 bp
CJ20 CJ21	L22	30		94°C, 1 min	50°C, 1 min	72°C, 1 min		600 bp

[a] For amplification of the 3 copies of the 23S rDNA, the same reverse primer (R-CJ) was used

DNA, as the *tet(O)* gene has been found to integrate into the chromosome.

2.5.5 PCR conditions for identification of mutations in the *gyrA* gene

The basis of ciprofloxacin resistance in *Campylobacter* isolates was determined by PCR amplification and purification of the *gyrA* gene product as previously described (151).

2.5.6 PCR for detection of *C. jejuni* associated virulence genes

Primers virB232 and virB701 designed by Datta *et al.* (30) were used to screen all plasmid preparations isolated from the 104 *C. jejuni* isolates for the presence of pVir (Table 2.9). Primers DMT 3 and DMT 4 were previously determined to be unable to amplify the *cjp5* gene by PCR (139). PCR for 10 putative virulence genes (Table 2.10) of *C. jejuni* was carried out as previously described by Datta *et al.* (30).

2.6 Growth competition of mixed cultures

C. jejuni strains NCTC11168 (streptomycin-resistant mutant [Str^R]) and *C. coli* isolates 001B-15 (erythromycin-resistant [Ery^R]) and UA585 (Ery^R), were grown on MH agar for 24 h at 37°C under microaerobic conditions. Bacterial cells were resuspended in MH broth and the OD_{600} adjusted to 0.20 ± 0.02 (10^8cells/mL) (58). These suspensions were used to inoculate 20 mL of MH broth to achieve 10^7 cells/mL of *C. coli* or *C. jejuni*. Mixed cultures were created by co-inoculating a *C. coli* isolate (001B-15 or UA585) and *C. jejuni* (NCTC11168) into MH broth at a 1:1 ratio. The actual number of bacteria present in the initial dilution was confirmed retrospectively by plating serial dilutions of the inoculum. Cultures were grown with shaking (150 rpm) for 72 h under microaerobic conditions at 37°C. Samples were removed from the culture at various time intervals over the 72 h period, OD_{600} values recorded, and appropriate dilutions plated onto MH agar containing either streptomycin (100 μg/mL) or erythromycin (16 μg/mL). Colonies were counted after plates were incubated for 48 h at 37°C under microaerobic conditions.

Table 2.8. Primers used in amplification and sequencing of the *tet(O)* gene. Adapted from Gibreel *et al.* (53).

Primer	Sequence (5'→3')	Purpose
DMT 1	GGCGTTTTGTTTATGTGCG	Amplification of 559bp
DMT 2	ATGGACAACCCGACAGAAGC	Amplification of 559bp
DOB 3	TATATGAATTCAATGAAAATAATTAACTTAGGCATTC[a]	Amplification and cloning of 1.92 kbp tet(*O*) gene (5' end)
SEAN 20	TATATGGATCCTCTAACTTGTGAACATATGCCG[b]	Amplification, cloning of 1.92 kbp tet(*O*) gene (3' end)
DMTS 27	GCAGACTTTCGGCTGCTTTC	Sequencing of tet(*O*) gene
DMT 29	GTGAAGCAAAAGGTTGGGCAGC	Sequencing of tet(*O*) gene
DMT 30	GCAGACTTTCGGCTGCTTTC	Sequencing of tet(*O*) gene
SEAN 6	CAGAACTGGAACTGGAACAGGAAG	Sequencing of tet(*O*) gene
JRU 9	GAGCTGTTGACAATTAATCATCGGC	Sequencing of tet(*O*) gene
JRU 10	CTGTTTTATCAGACCGCTTCTGC	Sequencing of tet(*O*) gene
SEAN 5	ACTGCTCCGTCTAATACG	Sequencing of tet(*O*) gene
SEAN 9	ATGCACCGCAGGAATATC	Sequencing of tet(*O*) gene

Restriction enzyme sites are underlined for [a]*Eco*RI and [b]*Bam*HI

Table 2.9. Primers used to amplify *cjp5* (*virB11*) in *C. jejuni* isolates and preparation of hybridization probe.

Primer	Sequence 5'→3'	Product Size (bp)	PCR 30 cycles		
			denaturation	annealing	extension
DMT 3	GAACAGGAAGTGGAAAAACTAGC	708	95°C (1 min)	50°C (1 min)	72°C (1 min)
DMT 4	TTCCGCATTGGGCTATATG				
virB232	TCTTGTGAGTTGCCTTACCCCTTTT	494	94°C (1 min)	53°C (1 min)	72°C (1 min)
virB701	CCTGCGTGTCCTGTGTTATTTACCCC				

Table 2.10. PCR primers and conditions for putative virulence genes of *Campylobacter jejuni*. Adapted from Datta *et al.* (30).

Target gene	Primer	Sequence (5'→3')	Product (bp)
cadF	cadF-F2B	TTGAAGGTAATTTAGATATG	400
	cadF-R1B	CTAATACCTAAAGTTGAAAC	
racR	racR-25	GATGATCCTGACTTTG	584
	racR-593	TCTCCTATTTTTACCC	
dnaJ	dnaJ-299	AAGGCTTTGGCTCATC	720
	dnaJ-1003	CTTTTTGTTCATCGTT	
ciaB	ciaB-403	TTTTTATCAGTCCTTA	986
	ciaB-1373	TTTCGGTATCATTAGC	
pldA	pldA-84	AAGCTTATGCGTTTTT	913
	pldA-981	TATAAGGCTTTCTCCA	
cdtA	DS-18	CCTTGTGATGCAAGCAATC	370
	DS-15	ACACTCCATTTGCTTTCTG	
cdtB	cdtB-113	CAGAAAGCAAATGGAGTGTT	620
	cdtB-713	AGCTAAAAGCGGTGGAGTAT	
cdtC	cdtC-192	CGATGAGTTAAAACAAAAAGATA	182
	cdtC-351	TTGGCATTATAGAAAATACAGTT	
wlaN	wlaN-DL39	TTAAGAGCAAGATATGAAGGTG	672
	wlaN-DL41	CCATTTGAATTGATATTTTTG	

2.7 Stability of erythromycin-resistance phenotype

Erythromycin MICs were determined following repeated subculture (15, 35, and 55 passages) on seven representative erythromycin -resistant isolates onto erythromycin-free MH agar plates with an E-test strip.

2.8 Efflux pump inhibitor assay

The effect of phenylalanine-arginine beta naphthylamide (PAβN, Aldrich, St. Louis, MO) on the erythromycin resistance of *Campylobacter* isolates was investigated as described previously (88) using standard susceptibility discs (Oxoid, Nepean, ON) containing 15 μg of erythromycin. Inhibition zone sizes were interpreted according to the guidelines of the CLSI. A control plate of MH agar containing an equivalent concentration of PAβN was also included to assess the effect of PAβN on the growth of the isolates.

2.9 Cloning of the *tet(O)* gene into the vector pMS119EH

The entire *tet(O)* gene (1.92 kbp) from *C. jejuni* isolates from poultry, was amplified from strains whose MIC values for tetracycline fell between 128 and 512 μg/mL (Table 2.8). The PCR products were then purified using a QiaQuick PCR Purification Kit (Qiagen, Mississauga, ON). The purified products were digested with *Eco*RI and *Bam*HI (Invitrogen, Burlington, ON). The plasmid vector pMS119EH was also digested with the enzymes. Both the digest of the gene and the plasmid vector were run on a 1% agarose gel, the bands of interest extracted, purified, and quantified using a spectrophotometer. Ligations were performed using T4 DNA ligase (New England Biolabs, Beverly, MA) with an insert to vector ratio of 3:1. Ligations were performed at 16°C overnight. The cloned pMS119EH:*tet(O)* was then transformed into *E.coli* DH5α, which had been made competent using a rubidium chloride preparation (See section 2.9.1).

2.9.1 Preparation of rubidium chloride competent DH5α

E. coli DH5α was grown in 100 mL of Luria Bertani (LB) broth (Difco, Beckton-Dickinson, Sparks, MA) at 37°C to an OD_{550}= 0.48. Cells were placed on ice for 15 min and then pelleted by centrifugation at 5000x*g* for 5 min The su-

pernatant was discarded and 40 mL of transfer buffer I (TfbI, 30 mM $KOOCCH_3$, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 15% v/v glycerol; pH 5.8) added. The cells were put on ice for 15 min and then pelleted at 5000x*g* for 5 min. The supernatant was again discarded and the cells resuspended in 4 mL of transfer buffer II (TfbII, 10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% v/v glycerol; pH 6.5). The DH5α cells were placed on ice for 15 min and then used immediately or stored in 500 μL aliquots at -80°C.

2.9.2 Transformation of pMS119EH:*tet(O)* into *E. coli* DH5α

To 10 μL of the ligated pMS119EH:*tet(O)*, 100 μL of rubidium chloride DH5α was added and subsequently placed on ice for 30 min. The cells were then subjected to heat shock (1.5 min) at 42°C and placed on ice for 2.5 min. LB broth (350 μL) was added to the cells and incubated for 1 h with shaking at 37°C. Aliquots of the suspension were then plated on ampicillin (100 μg/mL) LB plates and incubated at 37°C overnight. Colonies were picked and streaked onto LB plates containing 10 μg/mL of tetracycline and isopropyl-beta-D-thiogalactopyranoside (IPTG). Plates were incubated at 37°C overnight and then screened using a rapid phenol chloroform plasmid extraction method. Transformants were then plated onto tetracycline IPTG plates and incubated at 37°C overnight. Frozen glycerol stocks of each transformant were made and a loopful of the bacteria was also inoculated into LB broth containing tetracycline and IPTG, which was subsequently used to isolate the plasmid for sequencing.

2.9.3 Sequencing of *tet(O)* gene

The pMS119EH: *tet(O)* constructs were isolated and sequenced by a Big Dye terminator v3.0 Cycle sequencing kit (ABI Prism- A&B Biosystems , Foster City, CA) in the Molecular Biology Service Unit (MBSU, Department of Biological Sciences, University of Alberta) using several appropriate primers (Table 2.8).

2.10 Detection of pVir using DNA-DNA hybridization

2.10.1 Application of DNA to the membrane

Plasmid DNA (maximum of 250 μg) from the 104 *C. jejuni* poultry isolates, *C. jejuni* 81-176 (positive control), NCTC11168, and plasmids R27 and pMS119EH (negative controls) were spotted onto nitrocellulose paper (Invitrogen, Burlington, ON) and allowed to dry. The orientation of the nitrocellulose paper was marked by cutting off the top right corner of the sheet and the location of each plasmid sample recorded. The plasmid DNA was denatured in solution 1 (0.5 M NaOH, 0.15 M NaCl) for 5 min on a shaker. Nitrocellulose paper was blotted dry on a paper towel and placed in a drying oven for 5 min to remove excess liquid. Neutralization was performed by placing the membrane in solution 2 (10 mM Tris HCl, 0.15 M NaCl, pH7.5) for 5 min, blotted dry, and repeated once. Final wash was performed using 2 x SSPE (20x SSPE consists of 3.0 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA) for 5 min. The membranes were then placed between chromatography paper in a 65°C oven overnight.

2.10.2 Preparation of *cjp5/virB11* DNA probe

Primers DMT3 and DMT4 were used to amplify a 708 bp product from the pVir plasmid (Table 2.9) (9). A probe using the primers virB-232 and virB-701 for the *cjp5* gene were also used to amplify a 494 bp product (Table 2.9) (30).

The PCR product was purified using a PCR purification kit (Qiagen) and the DNA product was then eluted with TE buffer. A sample of the purified product was run on a 1% agarose gel to test purity and confirm the product size. The purified samples were diluted to a final concentration of 200 ng/μL in a final volume of 20 μL obtained using sterile Milli-Q water. DNA was denatured by boiling for 5 min and then placed directly on ice.

A random primer DNA labeling system (Gibco) was used to prepare the *cjp5* DNA probe. Added to the tube were 2 μL of dATP, dGTP, and dTTP, 15 μL of random primer buffer mixture, 3 μL of water, 5 μL of [α ^{32}P] dCTP, and 1 μL of Klenow fragment. The sample was centrifuged in an AccuSpin^{TM} microfuge, incubated at 25°C for 1 h and then boiled for 5 min to denature the DNA probe. All work with [α ^{32}P] dCTP was carried out behind a radiation shield. The ^{32}P

virB11 probe was used immediately for DNA-DNA hybridization experiments. Hybridizations were run with both probes to confirm the presence of the pVir plasmid.

2.10.3 Hybridization method for detection of pVir

The nitrocellulose paper was placed in the hybridization tubes and washed with 2x SSC (20x consists of 3.0 M NaCl, 0.3 M sodium citrate) for 5 min at 50°C in a Micro4 Incubation Chamber (Hybaid, Ashford, UK). The SSC solution was discarded and the hybridization solution added to the tube and mixed at 50°C for 2 h. The DNA hybridization solution consisted of 5x Dendhart solution [20x Dendhart solution consists of 1% wt/vol Ficoll 400 (Sigma), 1% wt/vol polyvinylpyrrolidone (Sigma) and 0.1% wt/vol bovine serum albumin], 0.1% sodium dodecyl sulfate (SDS; BioRad), and 6 x SSC and 100 μg/mL herring sperm DNA (Invitrogen, Burlington, ON). The *cjp5* probe was then added to the hybridization solution and incubated overnight at 50°C.

After overnight incubation with the radioactive probe, the nitrocellulose membranes were washed by incubating the blots with 0.5x SSC 15 min and repeated three times. The membranes were removed from the hybridization tubes and dried between sheets of chromatography paper. Membranes were then covered in plastic wrap and placed into a film cassette with BioMax MS film (Eastman Kodak Company, Rochester, NY) for 24 to 48 h at -80°C. A Kodak developing machine was used to develop the film.

2.11 Invasion assays using *C. jejuni*

2.11.1 *C. jejuni* isolates used in invasion assays

Twenty *C. jejuni* strains, selected following pulsed-field gel electrophoresis and *flaA*-RFLP analysis, were investigated for their ability to adhere to and invade Caco-2 cells (Table 2.11 and 2.12). A non-invasive *E.coli* K12 strain was included as a negative control for invasion. NCTC11168 was used as a control in each invasion study to ensure reproducibility between separate runs. The *C. jejuni* strain 81-176 was also investigated for its ability to invade Caco-2 cells.

A second set of *C. jejuni* isolates (n=20) was chosen based upon the presence or absence of blood in the stools of the patient from which the strain had been

collected. These isolates were obtained from the Alberta Provincial Laboratory for Public Health (APLPH). Preliminary invasion assays were completed in the same manner as for the other isolates.

Table 2.11. Isolates of *C. jejuni* from poultry used in invasion assays.

Isolate	*flaA*-RFLP Type	PFGE Type	pVir	pTet(O)
CJ412	41	13	-	+
CJ512	41	13c	+	+
CJ516	41	13d	+	+
CJ526	42	10h	+	+
CJ533	28	1d	-	-
CJ561	41a	24b	+	-
CJ574	13	6f	+	+
CJ632	18	8f	-	+
CJ655	41b	4	-	+
CJ687	13	5	-	+
CJ704	13	5	+	+
CJ713	21	5h	+	+
CJ755	18	8d	-	+
CJ809	28	1	+	+

Table 2.12. Isolates of *C. jejuni* from human stool samples used in invasion assays.

Isolate	*flaA*-RFLP Type	PFGE Type	pVir	pTet(O)	Presence of Blood in Stools
15-35	19	32a	-	+	-
16-08	18	8f	-	+	-
16-18	42	8h	-	+	unknown
16-70	24	10l	-	-	+
16-71	28	1d	-	-	-
23-69	18	8b	+	+	+
24-21	41	27b	-	+	-
24-28	41	25b	-	+	-
24-30	5	1	-	+	-
24-34	6	1f	-	+	+
24-48	25	38a	-	-	-
25-19	37	30a	+	+	+
25-29	25a	25c	-	-	+
25-44	22	19c	+	+	-
25-45	21	15d	+	+	-
25-50	23a	5m	+	+	-
25-53	5	17c	-	-	-
25-55	5	7c	+	+	+
25-61	14	32a	-	+	+
26-56	22	9i	-	-	-
27-05	28	17b	+	-	unknown
27-06	42	26c	-	+	-
27-44	45	16b	+	+	+
27-52	31	3a	-	-	-
27-57	3	34c	-	-	+
27-65	16	4f	-	-	+

2.11.2 Culturing of Caco-2 cells

Caco-2 cells (HTB-37) were obtained from the America Type Culture Collection (ATCC) (Atlanta, GA). The cells were grown in Eagle's Modified Essential Medium (EMEM, ATCC, Atlanta, GA) supplemented with 20% fetal bovine serum (FBS) (Fisher, Burlington, ON) without antibiotics at 37°C in a 5% CO_2 humidified incubator. For the experimental assay, Caco-2 cells were grown in 6 well plastic plates (Corning, Burlington, ON). The cells were seeded at $5x10^4$ cells per well and incubated for 14-16 days at 37°C in 20% FBS and 80% MEM, in 5% CO_2 humidified atmosphere. Prior to the assay cell monolayers were washed twice with phosphate buffer saline (PBS, pH 7.4).

2.11.3 Adherence and invasion assay

Campylobacter strains were grown microaerobically on MH agar plates for 16 h at 37°C. Bacteria were harvested from the plates and resuspended in EMEM including 1% FBS and adjusted spectrophotometrically to approximately $1x10^8$ bacteria per milliliter (OD_{600}=0.2±0.02). One milliliter of this suspension was inoculated into triplicate wells containing confluent monolayers of Caco-2 cells. The actual number of bacteria in the inoculum was determined retrospectively by serial dilution. Wells containing Caco-2 cells were trypsinized to determine the approximate number of cells present at the time of the invasion assay. Calculation of the multiplicity of infection (MOI) was determined to be $\sim$ 100 (ratio of bacteria:Caco-2 cell 100:1).

The wells of Caco-2 cells containing *Campylobacter* were incubated for 3 h at 37°C in a microaerobic atmosphere to allow bacterial adherence and invasion to occur. To determine adherence, cells were washed twice with PBS. Fresh medium without antibiotics was added to three of the wells. To the other set of wells, medium containing 250 μg/mL of gentamicin was added. The trays were then incubated for another 3 h to allow the gentamicin to kill the extracellular *Campylobacter*. Cells were treated with 1% TritonX 100 (Sigma, Madison, WI) for 5 min at 37°C. Serial dilutions were plated on MH agar and colonies counted after incubation of the plates for 48-72 h under microaerobic conditions.

Chapter 3

Results

3.1 Antimicrobial susceptibility of *Campylobacter* isolates

3.1.1 Erythromycin-resistant *C. jejuni* collection

The minimum inhibitory concentration (MIC) values for various antibiotics were determined for all *Campylobacter* isolates. For the 23 macrolide-resistant *C. jejuni* and *C. coli* isolates, susceptibility testing using agar dilution and E-test methodology was completed for erythromycin and clarithromycin. The MICs for erythromycin ranged from 128 to >1024 μg/mL. All isolates showed cross-resistance to the macrolide clarithromycin, with the exception of the clinical isolate 001A-114 (Table 3.1).

Of the 23 isolates, 17 were also found to be resistant to tetracycline using agar dilution. MIC values of the tetracycline-resistant isolates ranged from 16 to 512 μg/mL, with the highest MICs being demonstrated by the isolates collected from poultry (256 to 512 μg/mL) (Table 3.2). The incidence of tetracycline resistance among the isolates collected between 1974 and 1989 was lower than that of the isolates collected in Quebec between 2000 and 2001.

Two isolates (001A-15 and 001B-34) collected in 2001 were found to be resistant to >32 μg/mL of ciprofloxacin, an antibiotic which is sometimes used in the treatment of *Campylobacter* infections. None of the isolates collected between 1974 and 1989 were resistant to ciprofloxacin.

Table 3.1. Susceptibility of *Campylobacter* spp. isolates to macrolides.

Isolate	Erythromycin MIC (μg/mL)	Clarithromycin MIC (μg/mL)
C. coli		
001B-15	128	128
001A-18	256	128
UA585	>1024	128
001B-17	>1024	128
001A-15	>1024	128
UA37	>1024	128
UA609	>1024	64
UA749	>1024	128
C. jejuni		
UA40	256	64
001B-22	256	64
UA697	512	128
001A-168	512	64
001B-34	512	128
001B-31	512	64
001A-115	512	64
001A-114	512	2
UA695	>1024	64
UA709	>1024	128
001B-40	>1024	256
UA29	>1024	128
UA261	>1024	128
UA336	>1024	128
UA710	>1024	64

Table 3.2. Susceptibility of macrolide-resistant *Campylobacter* isolates to tetracycline.

Isolate	Tetracycline MIC (μg/mL)	Presence of *tetO* gene on a plasmid
C. coli		
UA585	<2	+
001A-18	32	+
UA749	32	+
UA609	64	+
UA37	128	+
001B-15	256	+
001B-17	512	+
001A-15	512	+
C. jejuni		
001A-168	< 2	+
UA697	< 2	+
UA336	< 2	+
UA710	< 2	+
UA40	< 2	-[b]
UA695	< 2	-[b]
UA709	16	-[a]
UA261	16	+
001B-31	32	+
001A-115	32	+
UA29	32	-[a]
001A-114	64	+
UA37	128	+
001B-40	256	+
001B-22	256	+
001B-34	512	+

[a] 559bp fragment amplified from total genomic preparation
[b] no product detected

3.1.2 *C. jejuni* isolates from Alberta poultry

Antibiotic-susceptibility testing of the 104 *C. jejuni* isolates obtained from poultry was completed using E-test strips, disc diffusion, and agar dilution. Of the 104 isolates, it was determined that 69% (72 of 104 isolates) were resistant to tetracycline using both the E-test and agar dilution methods. The disc diffusion method incorrectly classified four isolates as tetracycline susceptible (Figure 3.1). The agar dilution method determined that 55 of the tetracycline-resistant isolates had tetracycline MICs of 64 to 512 μg/mL and 9% (6 of 72) had a high level of resistance to tetracycline ranging from 256 to 512 μg/mL (Figure 3.2).

Sixty-four of the *C. jejuni* isolates from poultry were resistant to doxycycline according to all three methods (Figure 3.1). Of these isolates one was found to be resistant to 256 μg/mL of doxycycline and seven resistant to 128 μg/mL of doxycycline (Figure 3.2).

Of the 104 isolates tested, 11% (11 of 104) were resistant to nalidixic acid. The E-test failed to detect one of these nalidixic acid resistant isolates, whereas the disc diffusion method reported three isolates nalidixic acid-resistant when they were nalidixic acid-susceptible according to the other two susceptibility testing method (Figure 3.1). Seven of the isolates were found to have levels of resistance to nalidixic acid ranging from 128 to 256 μg/mL (Figure 3.2).

Ciprofloxacin resistance was observed in 8% of the isolates (8 of the 11 nalidixic acid-resistant isolates) (Figure 3.1). The E-test failed to detect one of the ciprofloxacin-resistant isolates, while disc diffusion incorrectly reported another isolate as resistant, when it was indicated as susceptible by the other methods (Figure 3.1). Four of the isolates were found to be resistant to a concentration of 32 μg/mL of ciprofloxacin (Figure 3.2).

All isolates were susceptible to amoxicillin-clavulanic acid, chloramphenicol, gentamicin, imipemen, and kanamycin. None of the isolates were resistant to erythromycin, the drug of choice in treating *C. jejuni* infections in humans.

Multi-drug resistant isolates were identified in this study. Six isolates were resistant to tetracycline, doxycycline and both fluoroquinolones (CJ449,CJ559, CJ604, CJ713, CJ714, CJ717). One isolate was resistant to the tetracyclines and nalidixic acid (CJ608). Two isolates were resistant to tetracycline and both fluoroquinolones (CJ412, CJ601) (Table 3.3). Susceptibility data on all 104 isolates used in this study are listed in the Appendix.

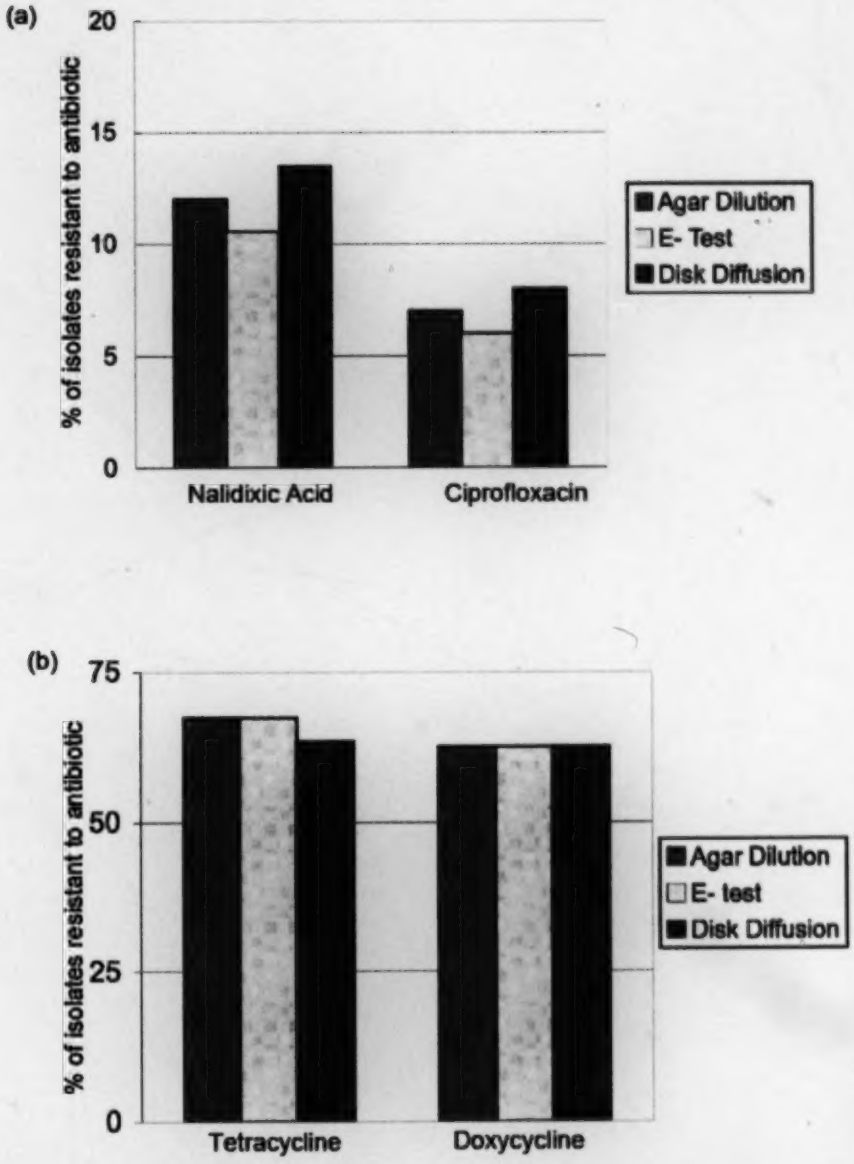
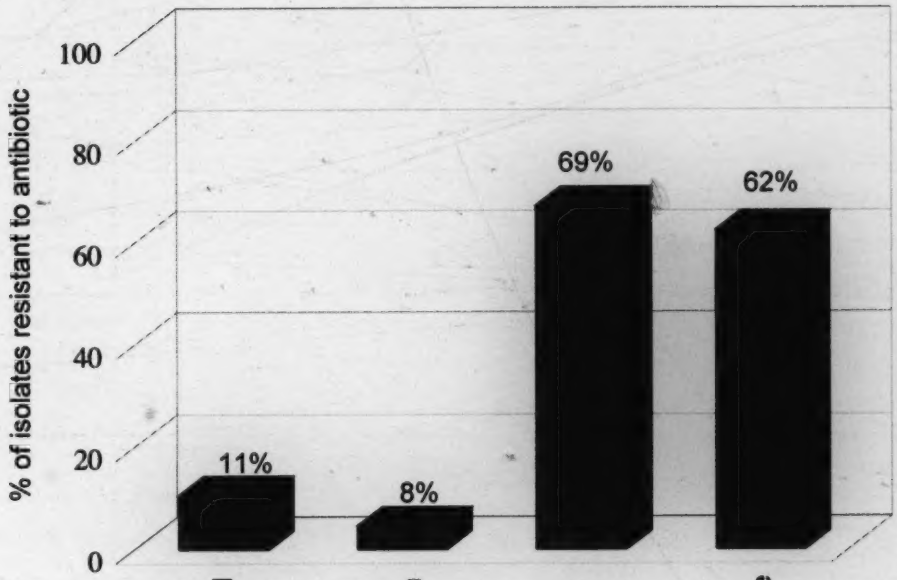


Figure 3.1. Antibiotic susceptibility testing of Campylobacter isolates by three different methods to (a) nalidixic acid and ciprofloxacin; (b) tetracycline and doxycyline.



MIC (mg/L)	Nalidixic acid	Ciprofloxacin	Tetracycline	Doxycycline
4		1		
8		1		
16		2	6	20
32		4	11	14
64	4		34	22
128	3		15	7
256	4		5	1
512			1	

Figure 3.2. Summary of antibiotic resistance present in *C. jejuni* poultry isolates (n=104) as determined by agar dilution.

Table 3.3. MICs of multi-drug resistant isolates of *C. jejuni* as determined by agar dilution.

Isolate	Tetracycline (μg/mL)	Doxycycline (μg/mL)	Nalidixic acid (μg/mL)	Ciprofloxacin (μg/mL)
CJ449	256	16	16	32
CJ559	64	64	64	32
CJ604	64	32	256	32
CJ713	32	16	256	16
CJ714	32	16	256	16
CJ717	128	16	256	4
CJ608	32	16	128	0.125
CJ412	256	0.016	64	32
CJ601	64	0.25	128	32

3.2 Characterization of resistance mechanisms

3.2.1 Screening for presence of *gyrA* gene mutations

The quinolone resistance determining-region (QRDR) of the *gyrA* gene of the highly ciprofloxacin-resistant isolates in both the erythromycin-resistance study (isolates 001A-15 and 001B-34) and the characterization of the *C. jejuni* isolates from poultry (CJ412, CJ449, CJ559, CJ601, CJ604, CJ713 and CJ714), showed the C-to-T transition at nucleotide position 256 resulting in the substitution of isoleucine for threonine at amino acid 86 which was previously observed to mediate quinolone resistance in *Campylobacter* (150).

3.2.2 Detection of the *tet(O)* gene

PCR amplification of a 559 bp region of the *tet(O)* gene, the resistance marker associated with conferring tetracycline-resistance in *Campylobacter*, was completed on all 23 *C. jejuni* and *C. coli* isolates used in the study of macrolide resistance and the 104 *C. jejuni* isolates from poultry.

3.2.2.1 Erythromycin-resistant *C. jejuni* collection

All 17 tetracycline-resistant isolates in the collection of the 23 erythromycin-resistant *Campylobacter* spp., were found to carry the *tet(O)* gene. Of those that were tetracycline susceptible, five contained plasmid DNA in which the Tet(O) determinant was detected. The other two contained the Tet(O) determinant in the chromosome. Therefore, 20 of the macrolide-resistant isolates were found to carry the gene on a plasmid; for two of the isolates the gene was detected in the total genomic DNA preparation, and for two, no *tet(O)* gene was detected (Table3.2).

3.2.2.2 *C. jejuni* isolates from Alberta poultry

PCR amplification of the *tet(O)* gene in the poultry isolates revealed that 67 of the 72 tetracycline-resistant isolates carried the gene on a plasmid. For the remaining five isolates, PCR analysis of total genomic DNA preparation revealed that the *tet(O)* gene was present in the chromosomal DNA.

The 559 bp fragment of the *tet(O)* gene was detected in 11 of the tetracycline-susceptible isolates (n=32). The PCR product was purified, sequenced, and aligned using the nBLAST search on the NCBI website (101). The sequenced product was found to align only with the *tet(O)* gene of *C. jejuni* (GenBank accession number M18896, published by Manavathu *et al.* (90)) and not with any other genes of the sequenced *C. jejuni* strains available (90, 109). The resistance level which the sequenced *tet(O)* gene confers in *C. jejuni* was determined to be to 64 μg/mL (90). Amplification of the full 1.92 kbp *tet(O)* gene was obtained from only one of these 11 tetracycline-susceptible isolates. In the remaining 10 isolates, amplification of the 1.92 kbp was unsuccessful.

3.2.3 Cloning of *tet(O)* from high-level tetracycline resistant isolates

Comparison of the sequences of the *tet(O)* gene of high-level tetracycline-resistant isolates (512 μg/mL) with that of the *tet(O)* gene cloned earlier by Manavathu *et al.* (89) revealed several differences (53). Modifications to the sequence of the *tet(O)* gene were made at a later point in time as sequencing of several *tet(O)* genes all showed a difference from the 3' terminal of the original sequence (53). Sequencing of the *tet(O)* gene in 21 high-level tetracycline-resistant *C. jejuni* isolates from Alberta poultry (MIC of 128 to 512 μg/mL), revealed that

the seven base changes, compared with the original sequenced isolate encoding a lower MIC, were present in three isolates with tetracycline MICs of 128 μg/mL (CJ758), 256 μg/mL (CJ625) and 512 μg/mL (CJ655). Six of the seven base changes were present in CJ617, which had an MIC of 128 μg/mL, with only the T910C transition absent. In 12 of the 15 isolates resistant to 128 μg/mL of tetracycline, a base change from C to T at position 704 occurred, which results in an amino acid change from an alanine to a valine, which are both nonpolar amino acids. The varying MICs of the strains with these base changes suggests that the substitutions may not be solely responsible for conferring high-level tetracycline resistance (512 μg/mL), although it may have some effect on Tet(O) determinants encoding MICs of 128-256 μg/mL (Table 3.4).

Table 3.4. Tetracycline (Tc) MICs of *C. jejuni* and base pair substitutions of the 1.92kb *tet(O)* gene of high-level tetracycline-resistant *C. jejuni* isolates compared to the sequence determined by Manavathu *et al.* (89).

Isolate	Tc MIC (µg/mL)	Mutations							
		Base pair	A884G	T910C	A993G	A1036C	T1063C	T1111G	A1784G	C704T
		Amino Acid	Y→C	S→P	I→M	I→L	S→P	C→G	Y→C	A→V
CJ655	512		+	+	+	+	+	+	+	-
CJ625	256		+	+	+	+	+	+	+	-
CJ758	128		+	+	+	+	+	+	+	-
CJ617	128		+	-	+	+	+	+	+	-
CJ755	128		-	-	-	-	-	-	-	+
CJ431	128		-	-	-	-	-	-	-	+
CJ432	128		-	-	-	-	-	-	-	+
CJ468	128		-	-	-	-	-	-	-	+
CJ494	128		-	-	-	-	-	-	-	+
CJ548	128		-	-	-	-	-	-	-	+
CJ570	128		-	-	-	-	-	-	-	+
CJ574	128		-	-	-	-	-	-	-	+
CJ666	128		-	-	-	-	-	-	-	+
CJ669	128		-	-	-	-	-	-	-	+
CJ717	128		-	-	-	-	-	-	-	+
CJ724	128		-	-	-	-	-	-	-	+
CJ730	128		-	-	-	-	-	-	-	-
CJ412	256		-	-	-	-	-	-	-	-
CJ449	256		-	-	-	-	-	-	-	-
CJ638	256		-	-	-	-	-	-	-	-
CJ762	256		-	-	-	-	-	-	-	-

3.2.4 Mechanism of macrolide resistance: sequencing of the 23S rDNA

Both *C. jejuni* and *C. coli* contain three copies of the 23S rDNA gene. Individual primers were designed to amplify separately, the three copies of the gene in *C. jejuni* as the genome sequence is available (109). As the genome sequence of *C. coli* is not available, the three copies could not be amplified individually. In this case, a set of primers designed to amplify a 300 bp region, where the mutation was located in *C. jejuni*, was amplified from the total genomic DNA preparation. The rational was that if a base pair change was observed, then a mutation was present in at least two copies of the 23S rDNA.

Sequence analysis revealed that all *C. coli* isolates exhibited an A→G transition at position 2059 (*E. coli* numbering). For *C. coli* isolate UA585, repeated amplification and DNA sequencing revealed that an A→G transition of the 23S rRNA gene may occur at either position 2058 or 2059 suggesting at least one of the three copies of the gene carries a different mutation (Table 3.5).

Sequencing of the 23S rDNA revealed that two of the 13 *C. jejuni* isolates exhibited an A→G transition at position 2059 in only two of the three copies of the 23S rRNA gene. Six of the 13 isolates showed an A→G transition at position 2059 in all three copies of the target gene while three isolates carried an A→C transversion at position 2058 in all three copies of 23S rDNA gene. In contrast, isolate 001A-168 exhibited an A→G transition at position 2058. Sequencing of the three copies of the 23S rDNA genes of UA710 revealed no point mutation at either position 2058 or 2059. Repeated attempts to amplify the three copies in UA261 yielded no products (Table 3.5).

3.2.5 Role of other ribosomal proteins in erythromycin resistance

Work on other bacteria, including *E. coli*, has suggested that mutation in L4 or L22 proteins which make up the large subunit of the ribosome could also be involved in mediating erythromycin resistance (154, 78). Two *C. coli* isolates (UA585 and 001B-15) and three isolates of *C. jejuni* (UA710, UA695, and 001B-22) exhibiting different mutations and varying levels of resistance to erythromycin, were examined for alterations in the ribosomal genes for L4 and L22. Two *C. jejuni* strains, NCTC11168 and 81-176, were included as representatives

Table 3.5. Mutations in 23S rDNA associated with macrolide resistance.

Isolate	23S rDNA position	Mutation[a]
C. coli		
001B-15	2059	A→G
001A-18	2059	A→G
UA585	2058/9	A→G
001B-17	2059	A→G
001A-15	2059	A→G
UA37	2059	A→G
UA609	2059	A→G
UA749	2059	A→G
C. jejuni		
UA40	2059	A→G
001B-22	2059	A→G[b]
UA697	2058	A→C
001A-168	2058	A→G
001B-34	2059	A→G
001B-31	2059	A→G
001A-115	2059	A→G
001A-114	2059	A→G
UA695	2058	A→C
UA709	2058	A→C
001B-40	2059	A→G
UA29	2059	A→G
UA261	2059	A→G[c]
UA336	2059	A→G[b]
UA710	2058/9	No mutation

[a] Mutation for *C. coli* based upon dominant genotype determined by sequencing of 300bp product. Mutation for *C. jejuni* determined to be present in all three copies of the 23S rDNA unless otherwise noted

[b] Mutation found in 2 copies

[c] Unable to amplify any of the 3 copies

of macrolide-susceptible strains. The L22 DNA sequence revealed that the protein of *C. jejuni* 81-176 has an insertion of six amino acids (118APAAKK) when compared to the L22 protein of NCTC11168.

The DNA sequences of the ribosomal proteins L4 and L22 of the five representative erythromycin resistant isolates demonstrated complete identity with the corresponding proteins of either *C. jejuni* NCTC11168 or 81-176 with the exception of the L22 protein in isolate UA695, which exhibited P13S alteration in the L22 protein. Isolate UA710 which was found to have no mutations in the 23S rDNA or in the L22 or L4 ribosomal proteins (sequence of UA710 aligned with that of NCTC11168) and suggests that other mechanisms are involved in mediating erythromycin resistance.

3.2.6 Role of efflux pumps in erythromycin-resistant *Campylobacter* spp.

PAβN has been reported to inhibit the CmeABC efflux pump of *Campylobacter* (88). It was found that adding this chemical would increase the organism's susceptibility to erythromycin (110, 88). The presence of 15 μg/mL of the PAβN inhibitor did not affect erythromycin resistance in 18 of the 23 isolates. One isolate of *C. jejuni* (001A-15) demonstrated an increase in the inhibition zone diameter for 15 μg of erythromycin from 0 to 45 mm in the presence of PAβN, indicating complete susceptibility to erythromycin. For *C. coli* isolates UA749 and 001B-15, the presence of PAβN resulted in a slight increase in the diameter of the inhibition zone from 0 to 22 mm and from 0 to 24 mm, respectively, although this was not sufficient to completely restore susceptibility to erythromycin (Table 3.6).

In two other isolates of *C. coli* (001B-17 and 001A-18), the presence of 15 μg/mL PAβN resulted in a marked inhibition of their growth (Table 3.6). The effect of two lower concentrations of PAβN inhibitor (5 and 10 μg/mL) on *C. coli* isolate 001A-18 had no influence on either its growth or level of erythromycin resistance. Although the presence of 10 μg/mL PAβN had no effect on the growth of isolate 001B-17, it did result in a slight increase in the inhibition zone diameter around the erythromycin disc, although it was not sufficient to restore complete susceptibility. The results suggest that the efflux pump plays a minimal role in mediating resistance to erythromycin.

Table 3.6. Effects of PAβN on the susceptibility of *Campylobacter* to erythromycin.

Isolate	PAßN (15 µg/mL)	Erythromycin (15 µg disc)[b]
UA749	-	0 (R)
	+	22 (I)
001B-15	-	0 (R)
	+	24 (I)
001A-15	-	0 (R)
	+	45 (S)
001B-17	-	0
	+	No growth[a]
001A-18	-	0
	+	No growth[a]

[a] Lower concentrations of PAβN did not affect the resistance of the isolates to erythromycin

[b] R= resistant
I= Intermediate
S= Susceptible

3.2.7 Stability of macrolide resistance

To determine if susceptibility could be lost with repeated subculture in the laboratory, seven representative isolates (001B-15, UA749, 001B-22, 001A-168, UA697, UA709, and UA40) were examined after repeated subcultures on drug-free medium after passage for 15, 35, and 55 days. Six of the isolates remained resistant to erythromycin. Isolate 001A-168 became susceptible to erythromycin (2 μg/mL) and clarithromycin (4 μg/mL) after 55 subcultures. Sequencing of the three copies of the 23S rRNA gene of the erythromycin-susceptible isolate 001A-168 revealed that position 2058 had reverted to wild type (G→A) and confirmed that the 2058 mutation was responsible for the original resistance phenotype.

3.3 Speciation of erythromycin-resistant *Campylobacter* isolates

A discrepancy in the identity of the isolates used in the study of erythromycin resistance arose when we were unable to amplify the three copies of the 23S rDNA

from an isolate which was classified as *C. jejuni*. From the literature available on methods for speciating *Campylobacter* spp., PCR for the *hipO* gene, *mapA* gene, *aspA* gene, and the hypervariable region of the 23S rDNA, were completed as outlined previously (20, 40). Results for 17 of the 23 isolates confirmed the species identity originally provided by the hippurate hydrolysis test (Table 3.7 and Table 3.8).

The results for some of the tests were in conflict with the original classification of some of the isolates (Table 3.8). Three of the isolates (UA261, UA336, and 001A-18) were originally misclassified, likely due to difficulties in reading the color change of the biochemical hippurate hydrolysis test. For isolates UA749, UA29, and UA40, discrepancies between the detection of the hydrolysis of hippurate and the presence of the *hipO* gene arose. Three isolates (UA29, 001A-15, and UA749) were suspected of being mixed cultures of *C. jejuni* and *C. coli* as the PCR methods specific for the detection of both species were positive. The 23S rDNA PCR-RFLP method generated confusion in the identity of three of the isolates (UA29, UA40, and 001B-40) suggesting that they were *C. jejuni* when the three PCR methods indicated that they were *C. coli*.

3.3.1 Multiplex-PCR analysis

The multiplex-PCR (m-PCR) correctly identified the *C. jejuni* and *C. coli* cultures producing a 735 bp (fragment of the *hipO* gene present in *C. jejuni*) and a 500 bp (fragment of the *aspA* gene present in *C. coli*) fragments respectively (129, 83). Although one of the *C. fetus* subsp. *venerealis* isolates, which served as a negative control, did produce a 380 bp fragment, this organism is not known to cause disease in humans (147). The m-PCR did not cross-react with any of the other *Campylobacter* spp. controls.

The frozen stocks of the isolates, where results of the individual PCRs suggested mixed cultures, were subjected to m-PCR. The m-PCR confirmed that the original frozen stocks of two of the isolates (001B-40 and 001A-15) were mixed cultures, while three other isolates (UA29, UA37, and UA40) were identified as *C. coli* (Table 3.8). When the frozen cultures of three of the isolates (UA29, 001A-15, and UA749) were plated and 15 single colonies of each isolate picked, m-PCR of the colonies suggested that only *C. coli* was present (Table 3.8).

Table 3.7. Species identification of *Campylobacter* isolates using the hippurate hydrolysis test and four molecular PCR methods.

Isolate	Hippurate hydrolysis[a]	PCR			23 S rDNA PCR-RFLP[c]	Final Classification
		hipO[a]	*mapA*[a]	*aspA*[b]		
001A-18	-	-	-	+	C	*C.coli*
001A-115	+	+	+	-	J	*C. jejuni*
001A-114	+	+	+	-	J	*C. jejuni*
001A-168	+	+	+	-	J	*C. jejuni*
UA609	-	-	-	+	C	*C. coli*
UA585	-	-	-	+	C	*C. coli*
UA 697	+	+	+	-	J	*C. jejuni*
UA695	+	+	+	-	J	*C. jejuni*
UA709	+	+	+	-	J	*C. jejuni*
UA710	+	+	+	-	J	*C. jejuni*
001B-17	-	-	-	+	C	*C. coli*
001B-15	-	-	-	+	C	*C. coli*
001B-34	+	+	+	-	J	*C. jejuni*
001B-31	+	+	+	-	J	*C. jejuni*
001B-22	+	+	+	-	J	*C. jejuni*

[a] + = *C. jejuni*
[b] += *C. coli*
[c] C= *C. coli*, J= *C. jejuni*

Table 3.8. Summary of speciation in *Campylobacter* isolates with discrepancies in identity as determined by different methods.

Isolate	Original Classification [a]	Year of isolation	Hippurate hydrolysis[b]	PCR				m-PCR		Corrected species Classification
				hipO[b]	*mapA*[b]	*aspA*[c]	23S rDNA[b]	Colonies[d]	Frozen stock[d]	
001A-15	*C. coli*	2000	+	+	-	+	C	C	C+J	Mixed
UA29	*C. coli*	1980	+	+	-	+	J	C	C	Mixed?
UA749	*C. coli*	1989	-	+	+	+	C	nc	nc	Mixed?
UA261	*C. coli*	1982	+	+	+	-	J	nc	nc	*C. jejuni*
UA336	*C. coli*	1982	+	+	+	-	J	nc	nc	*C. jejuni*
UA40	*C. coli*	1982	+	-	-	+	J	C	C	*C. coli*
001B-40	*C. coli*	2001	-	-	+	+	J	nc	C	*C. coli*
UA37	*C. coli*	1974	-	-	+	+	C	nc	C	*C. coli*
001A-18	*C. jejuni*	2000	-	-	-	+	C	nc	nc	*C. coli*

[a] Original classification determined by biochemical hippurate hydrolysis
[b] + = *C. jejuni*
[c] += *C. coli*
[d] C= *C. coli*, J= *C. jejuni*
nc= not completed

3.3.2 Growth of mixed cultures of *C. coli* and *C. jejuni*

As some of the isolates appeared to be mixed cultures of *C. jejuni* and *C. coli*, the growth characteristics of the two species were tested in an effort to determine if one species could out compete the other over time. The results indicated that there was no difference in the growth of the *Campylobacter* spp. in individual cultures versus mixed cultures (Figure 3.3). When *C. jejuni* isolate NCTC11168 and *C. coli* strain UA585 were grown in a mixed culture, UA585 was found to grow slightly slower than NCTC11168 but this was not due to the presence of the *C. jejuni* as the individual culture of UA585 (*C. coli*) was also found to grow slightly slower (Figure 3.3).

When plating strain UA585, a swarming phenotype was observed, making it difficult to count colonies on some occassions. An aliquot of the mixed culture was plated on non- selective medium, a loopful of this growth was plated onto a fresh non-selective MH plate, and then 10 single colonies picked, for m-PCR analysis. All colonies were found to be *C. coli*, illustrating that despite the slower growth rate of *C. coli*, other properties, such as its ability to swarm, allowed for it to out-compete the *C. jejuni* on the agar plate. Plasmid content did not have any obvious effect on the growth rate as illustrated by the growth rate of *C. coli* 001B-15 (Figure 3.3), which carries a plasmid encoding tetracycline resistance.

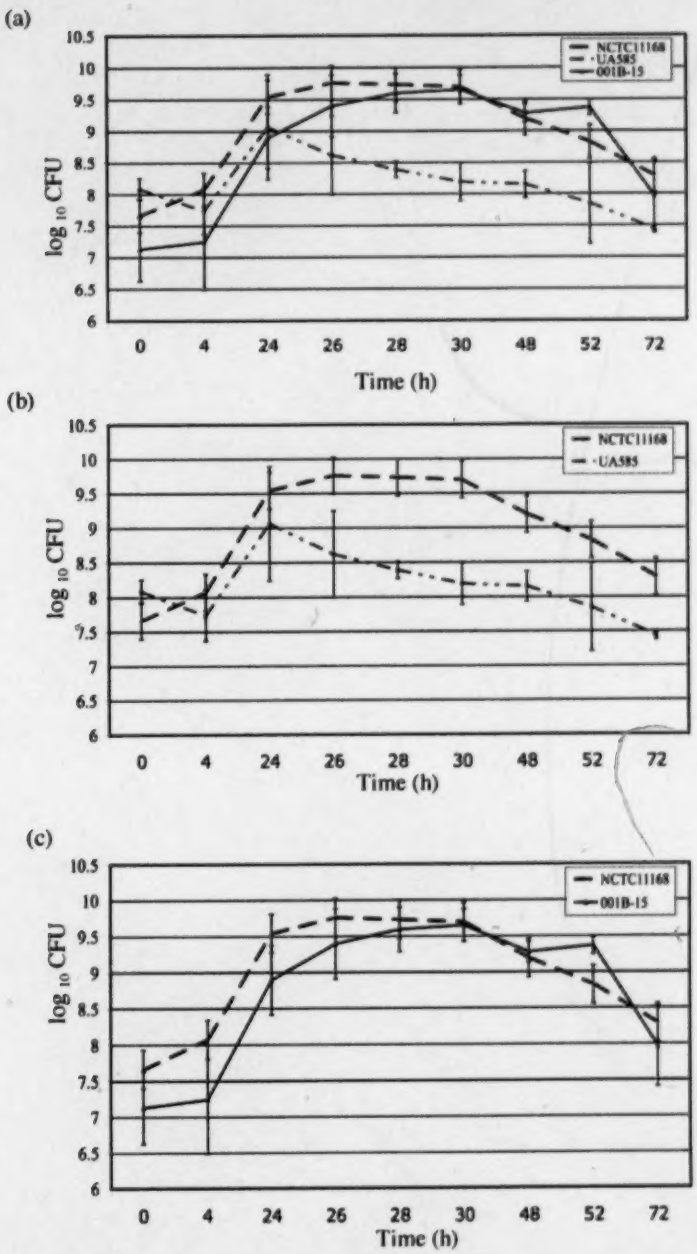


Figure 3.3. Growth curves of *C. jejuni* (NCTC11168) and *C. coli* (UA585 and 001B-15) as (a) individual cultures (b) mixed cultures of NCTC11168 and UA585 (1:1) and (c) mixed cultures of NCTC11168 (no plasmid) and 001B-15 (plasmid (1:1).

3.4 Presence of pVir in *C. jejuni* poultry isolates

The virulence plasmid, pVir, had previously been identified through DNA-DNA hybridization on colony blots of *C. jejuni* clinical isolates (9). Tracz (138) had attempted PCR using the primers designed by Bacon *et al.* (9) to confirm the presence of pVir in *C. jejuni*. Attempts were unsuccessful and the presence of pVir was confirmed using Southern hybridization instead.

In this study, purified plasmid DNA preparations were screened for the presence of pVir using two different probes in the Southern hybridization procedure. Probes previously used by Bacon *et al.* (9) and Datta *et al.* (30) were used in the Southern hybridization. The primers from Datta *et al.* (30) were also used to screen the plasmid preparations by PCR for a 494 bp fragment of the *cjp5* gene.

Southern blots using a ^{32}P-labelled *cjp5* probe from Bacon *et al.* (9) revealed that 65 of the 104 isolates (63%) contained the virulence plasmid. Southern hybridization using the probe from Datta *et al.* (30) confirmed the presence of pVir in 65 isolates.

Of the 65 isolates that contained pVir, 60 of them (92%) contained both pVir and pTetO. Of the remaining five isolates which contained only pVir, three of these were found to carry the *tet(O)* gene within the chromosomal DNA (CJ576, CJ591, and CJ634), indicating that although they once carried the autonomous plasmid it had subsequently integrated into the chromosome. The other two isolates (CJ561and CJ759) were tetracycline susceptible and harbored the pVir plasmid (Figure 3.4).

Results of PCR analysis using the Datta primers, agreed with those obtained by the Southern hybridization for the 65 isolates. However, discrepancies did arise with eight of the isolates. The Southern blots with either set of probes for the pVir plasmid did not hybridize with the DNA samples. When completing the PCR, a weak band of the expected 494 bp was observed, suggesting that the plasmid was present. Completing the hybridization again, still produced a negative result.

Digesting the plasmid preparations with *Bgl*II in an attempt to generate the pattern obtained for 81-176, a strain of *C. jejuni* in which the pVir plasmid was originally characterized (10), did not confirm the presence of the pVir plasmid in the isolates, with the exception of CJ542 for which on one occasion a *Bgl*II pattern identical to that of 81-176 was obtained. Similar discrepancies between Southern hybridization and PCR have been reported when screening for human papillomavirus (HPV) and it was suggested that this may be due to the stringency

of the the Southern hybridization combined with the small amount of viral DNA present in the sample (121).

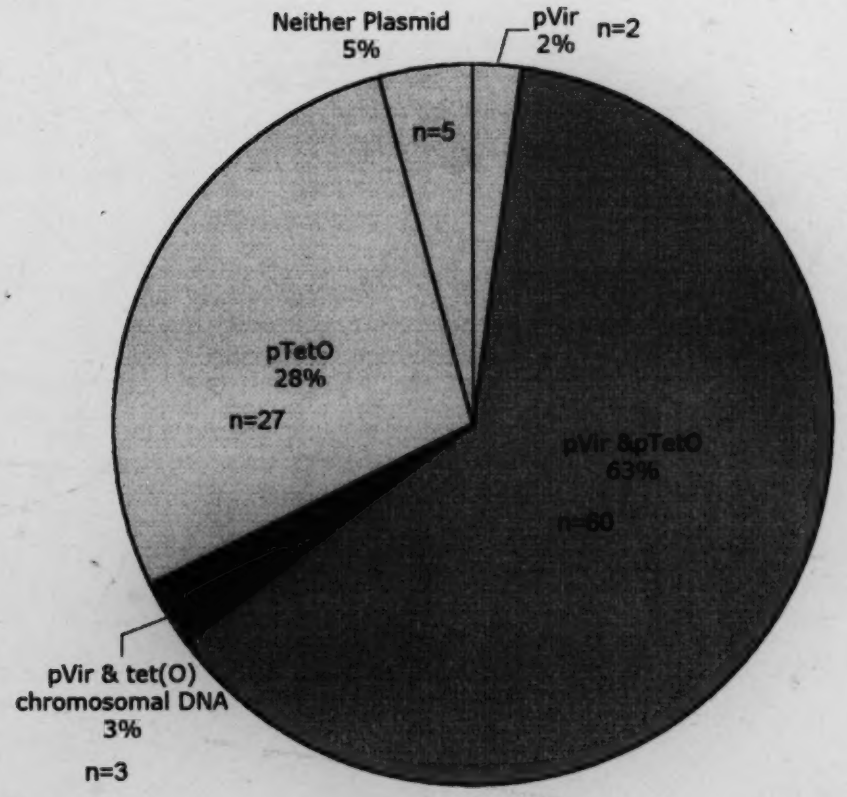


Figure 3.4. Plasmid content of 96 of the 104 *C. jejuni* isolated from poultry. The *tet(O)* gene was identified in plasmid preparations by PCR. The pVir plasmid was detected by Southern hybridization using two sets of probes and confirmed by PCR. Eight isolates are not included in this diagram as in their case, the Southern hybridization and PCR results did not agree.

3.5 Invasion assays of *C. jejuni* isolates using Caco-2 cells

The *C. jejuni* isolates used for the invasion assays were initially chosen based upon the relatedness found by *flaA*-RFLP typing and PFGE (Table 3.9). No correlation was found between the various patterns obtained by the typing methods and the ability of the isolates to invade the Caco-2 cells. Other characteristics of the isolates including the plasmid content, presence of putative virulence genes, and symptoms experience by patients from whom some of the strains were isolated, were examined and compared with the ability of the isolates to invade Caco-2 cells.

The invasion index and the percentage of adherence are shown for each isolate (Figure 3.5 and 3.6). Of the *C. jejuni* strains collected from poultry, CJ526, CJ412, and CJ687 each had an invasion index similar to that of NCTC11168 (Figure 3.6). Of the human isolates, 23-69 had an invasion index greater than that of NCTC11168 (Figure 3.6).

3.5.1 Analysis of *C. jejuni* invasion isolates for putative virulence genes

The isolates used in the invasion assays were screened for the presence of 10 genes associated with bacterial virulence (30). All of the isolates were found to have the *cadF, dnaJ, pldA,* and *flaA* genes. The *racA* gene was present in 91% (20 of 22) of the isolates. The *ciaB* gene was present in 95% (21 of the 22) isolates. The gene encoding cytolethal distending toxin (*cdtA*) was found in 64% of the isolates. The *cdtB* gene was found in 91% isolates. The *cdtC* gene was found in 77% of the isolates. The *wlaN* gene, which has been associated with strains isolated from patients who later develop GBS, was present in 12% (3 of the isolates) (Table 3.9).

No correlation between invasion and the presence or absence of the genes was determined as the virulence genes were present in most of the isolates. However, those isolates (CJ561, 24-28, and 27-05) which were found to lack at least two of the cytolethal distending toxin genes (*cdtC, cdtB* and *cdtA* genes) were found to have lower invasion indices than those isolates which had all of the toxin encoding genes. This toxin is known to be important for the entry of *C. jejuni* into host cells, and is effective when all three components of the toxin are produced (75).

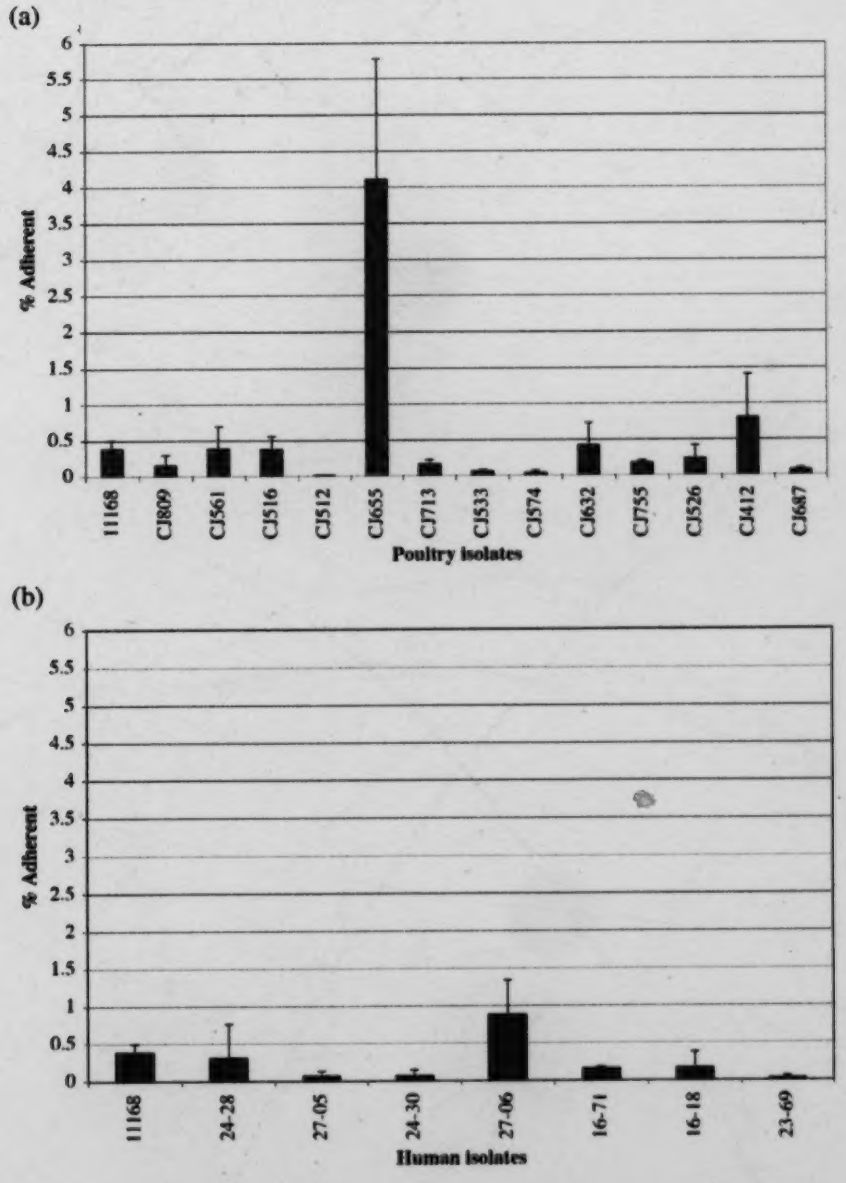


Figure 3.5. Adherence of *C. jejuni* isolates from (a) poultry and (b) humans to Caco-2 cells. Percent adherent is calculated by taking the number of extracellular bacteria divided by the inoculum, multiplied by 100. NCTC11168 is shown as a control which was run with each invasion assay.

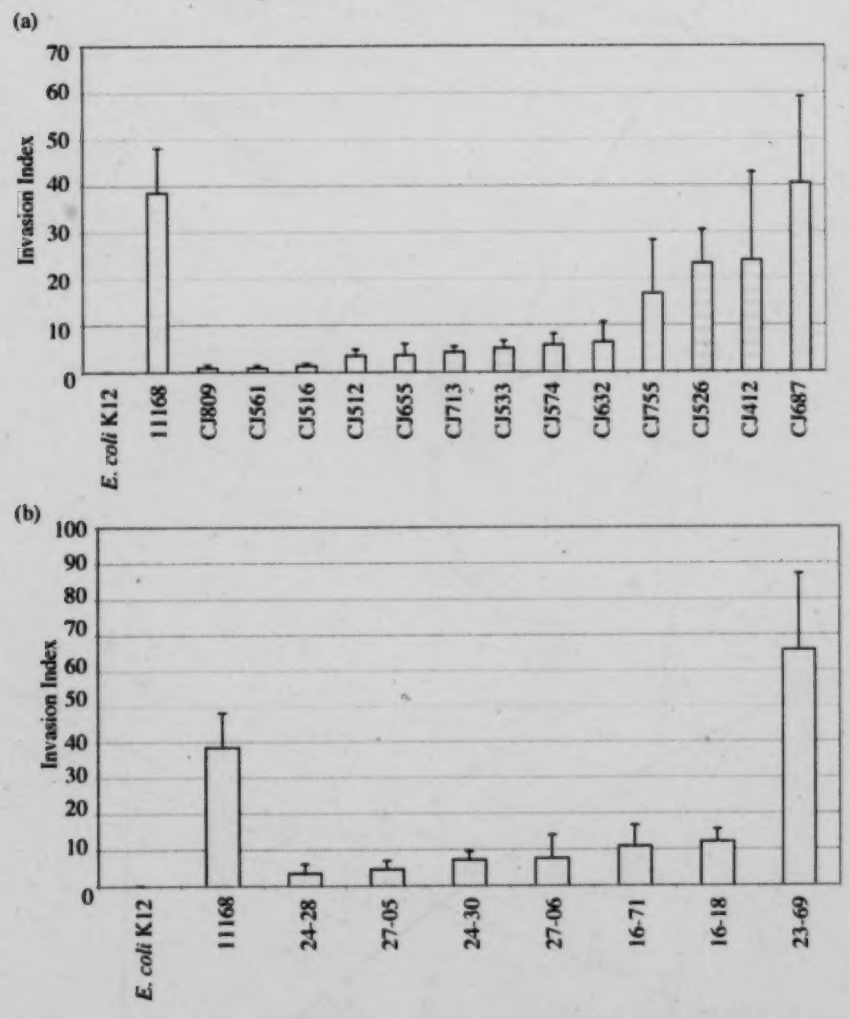


Figure 3.6. Invasion indices of *C. jejuni* isolates from (a) poultry and (b) human stool samples into differentiated Caco-2 cells. Invasion index is calculated as number of internalized bacteria divided by the number of adherent bacteria multiplied by 100. A strain of *E. coli* (K12) and NCTC11168 were run as negative and positive controls, respectively.

Although the genes may be present according to the PCR screening, it would be preferable to look at the expression levels of these genes, as the levels of each may affect how well the isolates invade the Caco-2 cells. Also, since the whole gene was not amplified, it is possible that the gene may not be functional in all of the isolates and may offer an explanation as to why varying levels of invasiveness were obtained.

3.5.2 Invasion ability of *C. jejuni* isolates with different plasmid profiles

It has been suggested that pVir plays a role in the ability of *C. jejuni* to invade INT407 cells (9). No correlation was found between the isolates containing plasmids, containing the *cjp5* gene or the *tet(O)* gene and the ability of the strain to invade in the Caco-2 cell model (Figure 3.7).

3.5.3 Comparison of invasion ability of *C. jejuni* isolates from patients with bloody and non-bloody stools

As no correlation between characteristics of the poultry isolates and human isolates were found, an attempt was made to correlate any clinical symptoms with the ability of the isolates to invade Caco-2 cells. Of particular interest was the presence or absence of blood in the stools of patients. A subset of clinical isolates of *C. jejuni* in which the presence or absence of blood in the stools had been reported, were chosen and invasion assays carried out. Due to time constraints, preliminary results from the assays are presented here. For the isolates in question, invasion assays have only been completed one or two times as this is still an on going study. The invasion index of isolates associated with the presence of blood in the stools was three fold higher than isolates from patients where no blood was reported (Figure 3.8). Statistical significance ($p<0.05$) was not achieved due to the small sample size. It is expected that the difference between the two groups may become statistically significant once more invasion assays are completed.

From this data, a possible model for choosing isolates to assess for virulence mechanisms may be developed. Isolates from other sources such as the poultry isolates, or from beef or water, may also be screened for their ability to invade Caco-2 cells. Highly invasive isolates may then be chosen and further screened using the ferret animal model, which could potentially reveal invasion mecha-

Table 3.9. Presence of 10 putative virulence genes and plasmids in 22 isolates of *C. jejuni*. Isolates are separated based upon source from which they were isolated. CJ isolates were obtained from poultry and numbered isolates from human stool samples. Two common *C. jejuni* strains were also screened for the presence of the putative virulence genes and are listed.

	Isolates from Poultry													Clinical Isolates							Reference Strains	
Isolate	CJ512	CJ655	CJ809	CJ516	CJ713	CJ561	CJ533	CJ632	CJ574	CJ755	CJ412	CJ687	CJ526	27-05	23-69	27-06	16-71	16-18	24-28	24-30	81-176	11168
Gene																						
flaA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cadF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
dnaJ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pldA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
racR	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
ciaB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
cdtC	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+
cdtB	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
cdtA	+	+	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	-	-	+	+	+
wlaN	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
Plasmid																						
pVir	+	-	+	+	+	+	-	-	+	-	-	-	+	+	+	+	-	-	-	-	+	-
pTetO	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	-
Typing																						
flaA-type	41	41b	28	21	41a	41a	28	18	13	18	41	13	42	28	18	42	28	42	41	5		
PFGE	13c	4	1	5h	24b	24b	1d	8f	6f	8d	13	5	10h	17b	8b	26c	1d	8h	25b	1		
Serotype	2	53	3	2	45	53	3	4	6	4	2	21	8	2	4	1	3	4	17	34		
HS O:							10	64	57	64		29	17	66	13 64 26	44	4 13	64	23 36			

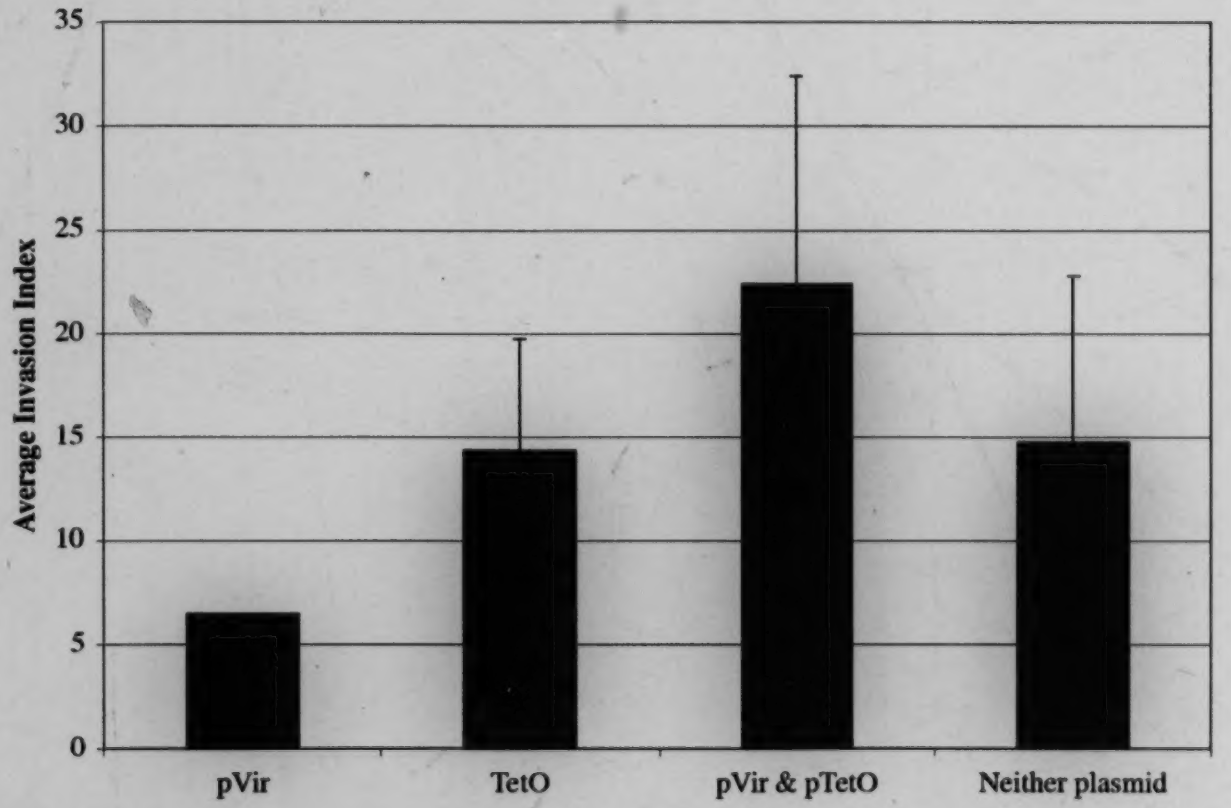


Figure 3.7. Relationship between presence or absence of plasmids in isolates of *C. jejuni* and the ability to invade Caco-2 cells. Values represent mean ± standard error of mean (SEM).

nisms they may use in causing infection in humans.

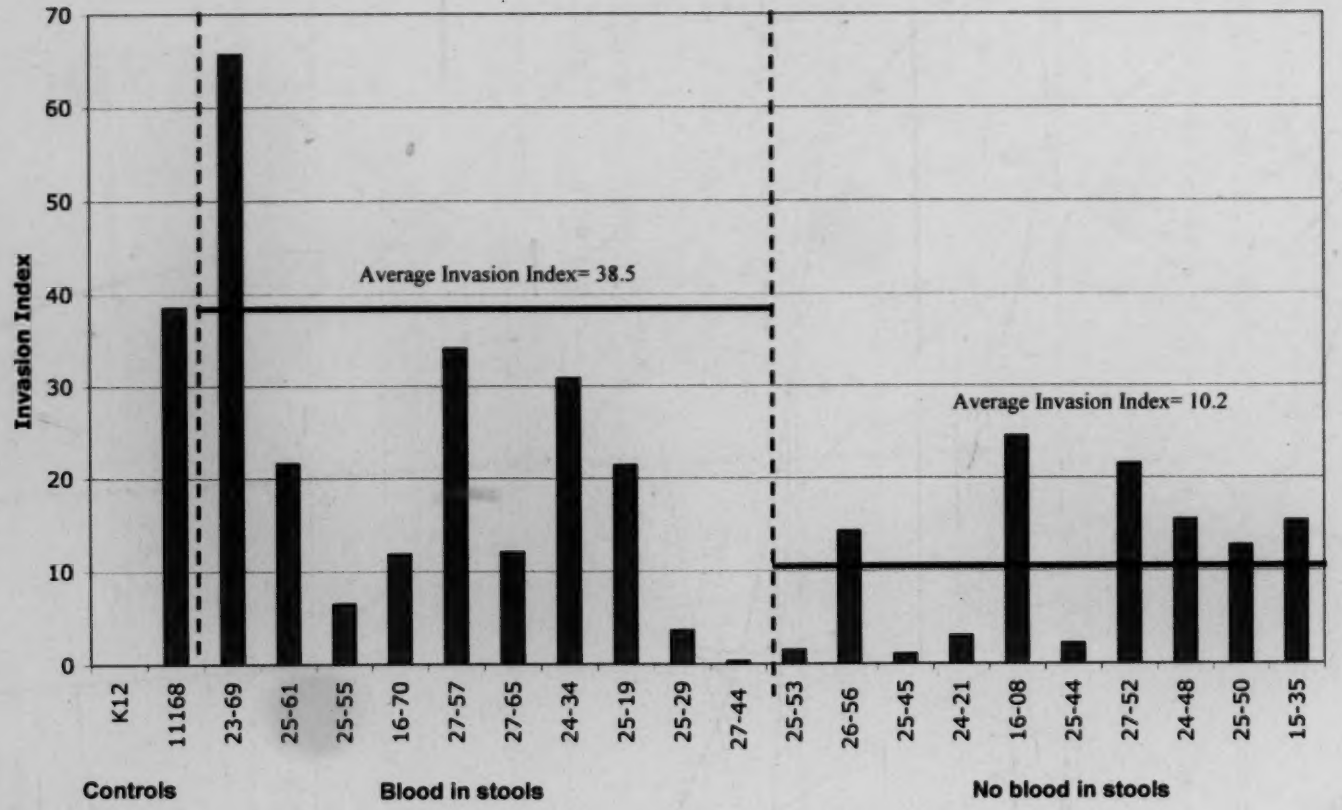


Figure 3.8. Preliminary results for invasion assays completed with isolates of *C. jejuni* from human stool samples in a Caco-2 cell model. Isolates were chosen based on the presence or absence of blood in the stools of the patient. Note that NCTC11168 and *E. coli* (K12) which served as controls are also shown. The average invasion index of the groups of isolates is also listed.

Chapter 4

Discussion

4.1 Antibiotic-resistance frequencies in *C. jejuni* isolated from poultry

Increasing resistance in *Campylobacter jejuni* is a concern as the development of multiple antibiotic-resistant strains jeopardizes effective antibiotic treatment in severe cases of infection. Antimicrobial resistance in *C. jejuni* has been reported to be increasing globally (4, 11, 48, 62, 117, 141). A recent study in Alberta, Canada, in which the antimicrobial susceptibility of *C. jejuni* clinical isolates was recorded, indicated that the incidence of tetracycline resistance had increased significantly to 49.8% compared to 8.6% in a similar study completed in 1981 (53, 135). Similar trends in the level of tetracycline resistance in *C. jejuni* were observed in Quebec (45).

The work in this thesis has provided a baseline for the levels of antibiotic resistance in *C. jejuni* present in Alberta poultry in 2001. Of the 104 isolates characterized in this study, all were found to be susceptible to amoxicillin-clavulanic acid, chloramphenicol, erythromycin, kanamycin, imipenem, and gentamicin. In addition, resistance to tetracycline (69%), doxycycline (62%), nalidixic acid (11%), and ciprofloxacin (8%) was observed.

The low frequency of resistance to ciprofloxacin and the absence of resistance to erythromycin is favorable from a clinical perspective as these two drugs are important for treatment of severe cases of *Campylobacter*-associated gastroenteritis. Antibiotic-resistance profiles of *C. jejuni* isolates in other countries demonstrate a much higher level of resistance to both ciprofloxacin and erythromycin (11, 85, 141). The appearance of ciprofloxacin resistance in the *C. jejuni* isolates

from poultry is significant as a recent study suggested that ciprofloxacin-resistant isolates are better fit and therefore preferentially selected in the gut of chicks without any antibiotic selective pressure (87).

The high frequency of tetracycline resistance seen in this study of poultry isolates is similar to those results found in a recent study, which characterized 203 clinical isolates in Alberta and determined that approximately 50% were resistant to tetracycline (53). Doxycycline is a tetracycline derivative used in agriculture. Acquisition of Tet(O) is usually associated with tetracycline resistance. It has been suggested that the *tet(O)* gene which confers resistance to tetracycline, is rapidly and spontaneously transferred *in vivo* without antimicrobial selection pressure between *C. jejuni* strains in the digestive tracts of chickens (7). This would provide, in part, an explanation for the somewhat higher frequency of tetracycline-resistant isolates from poultry compared with those from human infection.

Multi-drug resistance was low within the collection of *C. jejuni* strains from poultry, with only nine of the isolates being resistant to more than one class of antibiotic. An increase in the level of resistance to tetracycline and ciprofloxacin resistance was found to coincide in these multi-drug resistant isolates. No association was found between high tetracycline MICs and fluoroquinolone resistance as reported by Gaunt and Piddock (46) .

Antibiotic susceptibility testing standards for *C. jejuni* have only recently been set for certain antibiotics for *C. jejuni* (92). Values of antibiotic susceptibility and resistance have been suggested for a quality control strain are suggested for ciprofloxacin, doxycycline, erythromycin, gentamicin, and meropenem. It has been suggested by McDermott *et al.* (92), that a McFarland Standard of 0.5 be used as a reference when resuspending the *C. jejuni* isolates in MH broth. We found that using the 0.5 McFarland standard for antibiotic susceptibility testing for *C. jejuni* resulted in weak or no growth of the isolates, making it difficult to determine the E-test level or disc inhibition zone diameter. The use of a 1.0 McFarland reference, resulted in consistent results. To ensure that results obtained for the E-test and disc diffusion could be compared, 1.0 McFarland suspensions of *C. jejuni* were also used for the agar dilution testing.

During initial susceptibility testing it was also found that a number of the strains did not grow well on MH agar. Therefore, 5% defibrinated sheep blood was added to the medium and found to increase the viability of the strains, making the susceptibility testing clearer to interpret and more consistent. The use of this enriched medium is also recommended by McDermott *et al.* (92). The need

for enriched media and high inoculum density may be related to the source of the *C. jejuni* isolates. In the work of Gibreel *et al.* (53), the susceptibility testing of clinical *C. jejuni* isolates was completed using the 0.5 McFarland standard and MH agar and no difficulty in interpreting the results of disc diffusion were reported. Luber *et al.* (85) used a 1.0 McFarland standard as a reference, and although no comment was made on the use of this standard, the population of *C. jejuni* isolates tested in their study were obtained from both human and poultry sources.

Three different methods of antibiotic-susceptibility testing were used in this thesis to determine the MIC of each isolate. Although disc diffusion is an easy and affordable method, it was shown to over-estimate those isolates which were resistant in several cases. E-tests were found to be more reliable than disc diffusion and the results obtained by E-test agreed with the agar dilution method for tetracycline and doxycycline. With the fluorquinolones, E-test did miss one isolate when testing for resistance to nalidixic acid and ciprofloxacin, suggesting that it may not always be reliable in characterizing some isolates whose MICs are close to the breakpoint of these antibiotics. Observations in other studies have also suggested that the E-test is a suitable option for quick and easy determination of resistance levels of *C. jejuni* to certain antibiotics, allowing for a single $\log_2$ dilution variation from the agar dilution MIC results (47, 92).

4.2 Occurrence of the *tet(O)* gene in *C. jejuni* poultry isolates

The *tet(O)* gene was detected by PCR in all tetracycline-resistant isolates (72 of 104). A majority of the isolates were found to carry the *tet(O)* gene on a plasmid (67 of 72 isolates). This is similar to the findings of Gibreel *et al.* (53) where 67% (68 of 101) of the tetracycline-resistant clinical *C. jejuni* isolates were found to carry the *tet(O)* gene on a plasmid. It is interesting to note that the *tet(O)* gene appears to be plasmid-mediated in studies completed in Canada, while a recent study in Australia reported that the majority (76%) were found to contain the gene in the chromosomal DNA (115). Since both studies include isolates which were collected from humans and the location of the *tet(O)* gene confirmed by PCR and Southern hybridization, there is no clear explanation for the difference.

In this study, 21 isolates were found to have MICs of 128 to 512 μg/mL for

tetracycline. Previously several base pair changes within the *tet(O)* gene (A884G, T910C, A993G, A1036C, T1063C, T1111G, and A1784G) were found to confer tetracycline resistance in *C. jejuni* isolates to a level of 512 μg/mL. When the *tet(O)* gene was cloned into a plasmid vector and transferred to *E. coli* it conferred the same MIC to tetracycline (64 μg/mL) (132). Recent work in the Taylor lab, where the *tet(O)* gene from *C. jejuni* with MICs ranging from 64 μg/mL to 512 μg/mL is cloned and transferred to *E. coli*, confer a consistently low-level of resistance in *E. coli* (32 μg/mL). This may be due to inefficiency of the *Campylobacter* TetO protein to interact with the ribosome of the *E. coli* (53), and may offer a possible explanation as to why the *tetO* gene has never been detected as a mechanism of resistance in clinical tetracycline-resistant strains of *E. coli* (53) .

Sequencing of the 21 high-level *tet(O)* genes revealed that the same mutations were present in isolates resistant to 512 μg/mL, 256 μg/mL, and 128 μg/mL of tetracycline. One isolate determined to have a MIC of 128 μg/mL of tetracycline was also found to harbor six of the seven base pair changes, lacking the T910C transition. For the isolates resistant to a level of 128 μg/mL of tetracycline which possess the changes, it is possible that they are resistant to a higher level of tetracycline as it is accepted that the MIC determined by the agar dilution method could be one $\log_2$ dilution off. In previous work (53), no isolate resistant to a level of 256 μg/mL was sequenced. It is possible that the changes in the *tet(O)* gene begin at a concentration of 256 μg/mL.

The effects of such base pair changes in the Tet(O) protein are unknown as no crystal structure of Tet(O) is available. The Tet(O) protein shares homology with the elongation factor G (EF-G) of *Thermus thermophilus* for which the crystal structure is available (53). Localization of base substitutions in this homologous structure suggest that they are not consistent with changes in functional regions of the Tet(O) protein, although some of the exposed substitutions may be involved in protein interaction with the ribosome (27).

Host strain differences may also be a possible explanation for the varying levels of tetracycline resistance observed in isolates which carry the *tet(O)* gene. One possibility is the presence of an efflux pump. Within *C. jejuni*, two efflux pumps have currently been characterized and the genome is known to contain at least eight other non-resistance nodulation division (RND) efflux pumps (116). It may also be possible that mutations in other ribosomal proteins which interact with the Tet(O) protein may also exist and could confer higher levels of resistance.

Previous work on Tet(O) transformed into *E. coli* demonstrated that a mutation

in the *rspL* gene which encodes for the ribosomal protein S12, reduces the levels of tetracycline resistance (136). This suggests a possible link between the stability of the amino-acyl tRNA in the ribosomal site and tetracycline resistance mediated by the ribosomal protection proteins. Sequence analysis of the S12 ribosomal protein of *C. jejuni* isolates resistant to various levels of tetracycline may help explain these observations. Other ribosomal proteins such as S7 and S14 have been shown to mediate tetracycline resistance in other bacteria (136, 146), and the possibility remains that these ribosomal proteins may play some role in mediating tetracycline resistance in *Campylobacter* species. Although attempts at amplifying the S12 protein in the high-level tetracycline resistant *C. jejuni* isolates were made during the course of this thesis, no product was obtained. The primers did amplify DNA from the sequenced tetracycline-susceptible strain NCTC11168, suggesting that there is some type of mutation in these isolates or diversity in the genomes which could contribute to the varying levels of tetracycline resistance.

As PCR of the 559 bp fragment of the *tet(O)* gene began before the susceptibility level of the isolates to tetracycline were known, PCR was completed on the plasmid preparations of all of the isolates. For all isolates in which the *tet(O)* gene was not detected in the plasmid preparation, the total genomic DNA was subjected to PCR. This led to the identification of the *tet(O)* gene in 11 of the susceptible isolates. Sequencing of the 559 bp region confirmed that the *tet(O)* gene fragment was actually being amplified. Attempts to amplify the full 1.92 kbp of the *tet(O)* gene did not succeed, suggesting that the fully functional gene is absent possibly explaining why the isolates are susceptible to tetracycline. Further work in attempting to amplifying and sequencing the *tet(O)* gene in these susceptible isolates is required and could prove beneficial in understanding how the Tet(O) protein successfully mediates tetracycline resistance in *C. jejuni*.

In Alberta, there are no published data on antibiotic resistance of *C. jejuni* within animals. Such information is of interest as it could help determine the source of *C. jejuni* infections for humans and explain how the organism acquires resistance to a high-level of antibiotics. Although poultry is suspected to be the primary source of *C. jejuni* infections for humans, other sources such as water and cattle may also be important.

4.3 Mechanism of erythromycin resistance in *C. jejuni* and *C. coli*

The macrolide, erythromycin, is considered the drug of choice for treating serious *Campylobacter* infections (36). Macrolides are also used in the veterinary field for prophylactic and therapeutic purposes (36). The broad use of these drugs has raised concern regarding the development of antimicrobial-resistant strains. Erythromycin binds to the 50S subunit of the bacterial ribosome and interferes with protein synthesis by inhibiting the elongation of peptide chains (154).

In this study, the mechanism of macrolide resistance was determined to be due to a single point mutation in at least two of the three copies of the 23S rDNA. The most predominant mutation identified among *C. coli* and *C. jejuni* isolates was the A2059G mutation (*E. coli* numbering). The isolates exhibiting this mutation showed varying levels of resistance to erythromycin. The A2058C transversion, which also conferred erythromycin resistance, was found in only three of the *C. jejuni* isolates. The isolates with this mutation showed a narrow MIC range for erythromycin (512 to>1024 μg/mL).

The high frequency of the A2059G mutation among the isolates examined might be the result of biological features generated by this mutation. Growth experiments of isolates with the A2058C and A2059G mutation showed that they did have similar growth patterns, suggesting that the mutation offers no obvious advantage. It is possible that the A2058C mutation has minimal effects on the growth rate *in vitro*, but that the effects may be more important *in vivo* over a long period of time.

In one of the *C. jejuni* isolates (001A-168), an A2058G transversion was observed. This mutation is homologous to that seen in macrolide-resistant *H. pylori* (A2142G) (32). The rarity of this mutation may be explained by the results of the experiment examining the stability of the various erythromycin mutations. After 55 days of growth on non-selective media, the susceptibility of strain 001A-168 to erythromycin was restored and the mutation was no longer detectable. It should also be noted that as a result of the instability of this mutation, it may be underestimated in the population.

Inability to amplify the three copies of the 23SrDNA in *C. jejuni* strain UA261, suggests that the ribosomal RNA operons in some *C. jejuni* isolates may exhibit a certain degree of sequence variation at the position where the primers for the 23S rDNA bind, resulting in no PCR amplifications.

The results of the study of the three copies of the 23S rDNA and the role they play in conferring resistance to erythromycin, suggests that at least two copies must carry the mutation to confer macrolide resistance. However, unlike in previous studies (66, 110), no correlation was found between the number or position of the mutation and the level of erythromycin resistance.

In other bacteria, including *E. coli* and *Streptococcus* spp., mutations in the ribosomal proteins L4 and L22 have been implicated in macrolide resistance (78, 154). Sequencing of the genes encoding these two proteins in *C. jejuni* (UA710, 001B-22, & UA695) and *C. coli* (UA585 & 001B-15), did not provide any evidence for the involvement of these two proteins, as the sequence of the resistant isolates was identical to that of the L4- and L22- encoding genes of erythromycin-susceptible strains (NCTC11168 and 81-176).

The efflux pump inhibitor, PAβN, was found to potentiate the effect of erythromycin in four cases but complete susceptibility was restored only in isolate 001A-15. It is not clear why the isolate became susceptible even though it still contained the A2059G mutation in all three copies of the target gene. A similar observation was made in a study of the effects of an efflux pump inhibitor on the susceptibility of *Pseudomonas aeruginosa* to levofloxacin. The presence of the target-based mutation did not confer resistance in the presence of the inhibitor (84). The use of PAβN was chosen to inhibit the efflux pump in this study as it had been used previously for *C. jejuni* (88). Some of the isolates were unable to grow in the presence of the 15 μg/mL of PAβN. However, higher concentrations of the inhibitor (40 μg/mL) of PAβN have been used and have not been reported to affect the growth of the isolates examined (110). This would suggest that the effect of PAβN on the growth of *Campylobacter* could be isolate dependent and may require optimization before it can be shown to directly affect efflux.

Despite screening for mutations in various genes which would be involved in mediating erythromycin resistance in *Campylobacter* spp., no efflux pump, mutation in the ribosomal proteins L4 and L22, or base change in the 23S rDNA gene were detected in *C. jejuni* isolate UA710. This finding would suggest that there is another mechanism involved in mediating the resistance in this isolate. Mutations at other positions in the 23S rDNA associated with macrolide resistance, including position 2611 (96) and 2717 (42), were not detected in any of the copies of the target gene. The possibility that UA710 was producing an extracellular enzyme capable of modifying erythromycin was also considered. The assay indicated that extracellular degradation of erythromycin was not responsible for

conferring macrolide resistance in isolate UA710. Although no further possible mechanisms were explored in this study, there exists the potential that another ribosomal protein, or another uncharacterized efflux pump may contribute to the resistance phenotype of strain UA710.

4.4 Identification of *C. jejuni* and *C. coli* species

C. jejuni and the closely related *C. coli* have been recognized as the leading causes of bacterial foodborne diarrheal disease in humans (21). Greater than 95% of *Campylobacter* infections are caused by these two species (21). Precise species identification is increasingly important in understanding the disease spectrum, tracing sources of infection, and determining the routes of transmission of *C. jejuni* and *C. coli*. To speciate *Campylobacter*, the standard biochemical and phenotypic tests performed include: catalase production, hippurate and indoxyl acetate hydrolysis, H_2S production in triple sugar agar, and susceptibility testing with nalidixic acid and cephalothin.

The ability to hydrolyze hippurate, due to the presence of the enzyme hippuricase (encoded by the *hipO* gene) is unique to *C. jejuni*. Moreover, it is the only biochemical test which can be used to differentiate *C. jejuni* from *C. coli* (hippurate-hydrolysis test negative). However, hippurate hydrolysis-negative *C. jejuni* isolates have been reported due to the truncation, or low expression of the *hipO* gene (40, 137). Detection of the *hipO* gene by PCR is considered confirmatory of *C. jejuni* as the gene is highly conserved among *C. jejuni* isolates (125). It is generally accepted that a negative hippurate hydrolysis test may not be used to definitively identify *C. coli* (34, 137). When the *hipO* gene is detected in hippurate hydrolysis-negative isolates, they should be identified as *C. jejuni*. Accordingly, PCR methods provide an easy, reliable, and reproducible approach to differentiating *Campylobacter* species although these methods are associated with increased cost and time (5, 20, 34, 40, 128, 137, 148, 149). A high through-put screening system which minimizes the cost of reagents, tests and time to speciate clinical isolates of *Campylobacter* has been suggested (77).

In this study, the biochemical hippurate hydrolysis test alone was found to be insufficient for detecting *C. jejuni*. In contrast, PCR to detect the *hipO* gene was considered a reliable indicator of the presence of *C. jejuni* in the cultures. Moreover, the presence of the *mapA* gene was not reliable for differentiation between *C. jejuni* and *C. coli* isolates as it was found to be non-specific. Difficulties with

its specificity have been reported previously (106). A discrepancy arose upon comparison of the 23S rDNA PCR-RFLP with the original identity of the isolates.

There are a multitude of PCR methods available for the speciation of the two closely related *Campylobacter* species, making it time consuming and expensive to evaluate each one. Therefore individual laboratories generally chose one or two tests to differentiate the two species. In this study, the *hipO* and *aspA* PCR were found to be reliable PCR methods, and allowed for a way to positively differentiate *C. jejuni* from *C. coli*. Concurrent amplification of the two genes was attempted as the two sets of primers appeared to be ideal candidates for a m-PCR because the products were similar in length and the annealing temperatures were close. The m-PCR worked well as it consistently amplified only the expected products in the two *Campylobacter* species.

Investigation into the growth of *C. jejuni* and *C. coli* was pursued when it was determined that mixed cultures of the two species existed. However, in completing the m-PCR and single amplifications of the *hipO* and *aspA* gene on colonies picked from three different isolates, only one of the species was detected. Although various combinations of *C. jejuni* and *C. coli* were examined, no significant difference between the growth-rate of the two species was observed. A plausible explanation is that one species in the mixed culture may be more suited to survive the freezing and thawing process to which the stocks are subject although, this hypothesis was not investigated further.

Although the cost of confirming the species of the isolates increases with the addition of PCR, determining the *Campylobacter* species involved in the infection is important for understanding the epidemiology and disease relevance of both *C. jejuni* and *C. coli*. This thesis also demonstrates that mixed cultures of erythromycin-resistant *C. coli* and *C. jejuni* may exist *in vivo*. Although transfer of macrolide resistance has been demonstrated *in vitro* (49), it is not clear if this occurs *in vivo* either between the same species or between related species.

4.5 Virulence plasmid pVir and its frequency in *C. jejuni* isolated from Alberta poultry

A number of enteric pathogens including *Salmonella*, *Shigella*, and *E. coli* contain plasmids which are known to encode proteins which contribute to the pathogenesis of these organisms in humans. In *C. jejuni* a plasmid, pVir, is be-

lieved to be involved in virulence (9, 10). The plasmid pVir was isolated from *C. jejuni* strain 81-176, which was originally isolated from an outbreak of *C. jejuni* associated with unpasteurized milk (73). The plasmid is $\sim$ 37.5kB in size and has been found to contain genes sharing homology to those found in a type IV secretion system (T4SS). The role of T4SS is well documented and is known to be important for the virulence of a number of major bacterial pathogens including *H. pylori* (25).

In vitro, pVir has been suggested to be important both in adherence and invasion in INT407 tissue culture cells as well as in the ferret model (9). The gene believed to play a major role in the virulence of *C. jejuni* on this plasmid is *cjp5*, a *virB11* homolog. In the study completed by Bacon *et al.* (9), 10% of fresh clinical isolates of *C. jejuni* (n=58) were determined to contain pVir by completing DNA-DNA hybridization of colony blots with a ^{32}P-labelled *cjp5* DNA probe.

A recent study of clinical isolates from Alberta found that 15% (21 of 137) clinical isolates contained the pVir plasmid (139). Similar frequencies of the plasmid in *Campylobacter* isolates have been reported (9, 30, 122). Various methods have been used to determine the presence of pVir (9, 30, 139).

In this thesis, plasmids were isolated from 104 *C. jejuni* by using HiSpeed Maxi Plasmid Isolation Kits (Qiagen) and hybridization completed with two different probes (9, 30) which had been designed to detect the *cjp5* gene. It was found that one of the primers for the *cjp5* gene from Bacon *et al.* (9) bound within the *cjp5* region where as the other was found to bind within a non-specific region. In contrast, the primers for the *cjp5* gene designed by Datta *et al.* (30) both aligned to regions within the *cjp5* gene. PCR of the plasmid preparations was attempted with both sets of primers. The *cjp5* primers designed by Bacon *et al.* (9) did not amplify the gene segment (708bp) in several isolates tested and this result was confirmed by work completed earlier (139). This is believed to be due to the fact that one of the primers binds external to the gene and that this region may vary in sequence in plasmids from individual isolates. The *cjp5* primers from Datta *et al.* (30) did amplify a 494bp fragment of the *cjp5* gene from the isolates confirmed to contain pVir by Southern hybridization.

Of the 104 *C. jejuni* poultry isolates, 65 isolates (63%) were found to contain the pVir plasmid. This is a much higher percentage of isolates when compared to other findings in the literature. Nevertheless, this is the first study in which such a large sample of *C. jejuni* isolates obtained from poultry have been screened for the presence of this plasmid. Little is known about the pVir plasmid regarding

similarity between isolates or copy number.

Another possible reason to explain the apparent high percentage of pVir plasmid positive isolates could be the presence of another plasmid sharing similarity in the *virB11* gene which may be more prevalent in the *C. jejuni* population in poultry. It has recently been suggested that *C. jejuni* carry subgroups of related plasmids ranging in size from 36kb to 66 kb that carry genes which share homology to those found in the type four secretion system (122). However, when the plasmids were digested with *Bgl*II, distinct patterns from that of pVir from 81-176 were present (122).

The difference in the number of isolates found to contain the pVir is interesting from the perspective that it is believed that poultry are a major source of *C. jejuni* gastroenteritis for humans. However, the difference in the pVir plasmid content of the *C. jejuni* from humans and poultry, suggests that two distinct populations exist. Consequently *C. jejuni* infections may be acquired from another source such as water or beef rather than primarily poultry. Another possibility is that the bacteria undergo a change when entering the gut of the human host, which involves the loss of the plasmid. Further investigations are required into the role that this plasmid plays in the ability of *C. jejuni* to successfully establish and cause infection within a human host.

4.6 Invasion of Caco-2 cells

Studies of host-pathogen interactions often involve the use of cultures of epithelial origin *in vitro*. *C. jejuni* is known to infect the small intestine of the human host. These cells are polarized with distinct apical and basolateral membranes. *In vitro*, there are a number of cell lines that have been established for the study of enteric pathogens. *C. jejuni* has been studied using HEp2, HeLa, INT407 and Caco-2 cells (9, 22, 58, 71, 80). The Caco-2 cells are considered to be an appropriate model for studying *C. jejuni* invasion and adherence. They develop enterocytic morphology after 6 days, characterized by the formation of a brush border containing microvilli, the formation of occluding junctions at the apical surface, and a tall compact shape (44). Caco-2 cells develop fully differentiated columnar cell monolayers between 14 and 21 days in culture (44, 130). The appearance of dome like cells at this stage suggest that the Caco-2 cell monolayer is functionally polarized (114).

Recent studies involving *C. jejuni* and Caco-2 cells at 6 days old, where the

cells have begun to take on enterocytic morphology but are not completely differentiated, have been published (22, 58). A recent publication suggested that a standard method for carrying out invasion assays for *Campylobacter* be established (44). There are a number of studies in which invasion assays using different cell lines and discrepancies in how the results are reported make it difficult to compare results between different studies.

In completing a study of the invasion ability of *C. jejuni* strains, it is also important to consider the characteristics of the individual strains used as much as the cell model. There is widespread genetic diversity among the *Campylobacter* genus and within *Campylobacter* species. Studies on the genetic structure of *C. jejuni* populations are inconclusive and it is unknown whether there are distinct clonal lineages of pathogenic types (94). It has been suggested that there is a variation in the invasive ability of various *C. jejuni* due to a variation in the virulence of *C. jejuni* cells (9). The existence of subpopulations of *C. jejuni* with different mechanisms of pathogenesis would be equivalent to the various serotypes of pathogenic *E. coli*.

In this thesis a cell model using differentiated Caco-2 cells grown for 14-16 days was used to explore the invasion abilities of 22 different strains of *C. jejuni*. The *C. jejuni* used were chosen based upon the similarity seen in the *flaA*-RFLP and PFGE profiles. The isolates were of clinical and poultry origin. PCR analysis for the presence of putative virulence genes, pVir and pTet(O) was also determined. No association was found between the invasion ability of a strain into Caco-2 cells and the characteristics of the strains in regards to the pulsed-field type, associated virulence genes, or presence of plasmids.

Given the limited information available on the isolates of *C. jejuni* obtained from poultry, another subset of *C. jejuni* isolates (n=20) which had been associated with human infection and for which the clinical symptoms were available, were used in invasion assays involving Caco-2 cells. Symptoms associated with these isolates were available and compared with the invasion data collected. The average invasion index observed for the isolates obtained from patients who had reported bloody diarrhea associated with the infection were three fold higher than for those strains from patients who did not report this symptom. This preliminary work suggests that the Caco-2 cell model is an appropriate model for comparing the invasive ability of isolates and the severity of gastroenteritis (using blood in stools as an indicator of severity).

Comparison of the clinical data associated with *C. jejuni* infections in humans,

would appear to be a way to determine the virulence mechanisms necessary for a *C. jejuni* strain to cause disease in humans. Using the symptoms (such as blood in the stools), isolates of interest may be selected and used in invasion assays using Caco-2 cells. Such experiments might include examining the isolate for expression of putative virulence genes or analysis of virulence in the ferret model.

Currently there is a move towards establishing a standard *in vitro* model system for the study of *C. jejuni* (44). Recent studies using Caco-2 cells suggest that this cell model is appropriate for determining how well isolates colonize the intestines of poultry (58). From preliminary data collected, it would appear that the Caco-2 cell model is also useful for measuring the invasion ability in human infection. Further work is required in order to establish the Caco-2 cell model in our lab. One improvement would be to have an invasive *Salmonella* spp. as a positive control for invasion. Potentially looking at growing the Caco-2 cells on transwell filters is another possible way to improve the success of the 14 day model. Having an *in vitro* cell model, which could potentially provide information concerning both the clinical and environmental aspects of *C. jejuni* would be most appropriate. Such a model could help explain the link between environmental sources of *C. jejuni* and the route of infection in humans as well as the mechanisms *C.jejuni* uses to establish an infection within humans.

4.7 General Conclusions

This thesis reports the antimicrobial-resistance profiles of *C. jejuni* isolated from poultry in Alberta, and correlation with *tet(O)*, the gene encoding tetracycline resistance. All *C. jejuni* isolates were susceptible to amoxicillin-clavulanic acid, chloramphenicol, erythromycin, gentamicin, imipemen and kanamycin. The observation of erythromycin susceptibility in all *C. jejuni* poultry isolates studied is a beneficial finding as erythromycin is the drug of choice in treating human *C. jejuni* infections. In contrast, the observation of ciprofloxacin resistance in some *C. jejuni* poultry isolates, is an important finding since it is also prescribed for the treatment of human *C. jejuni* infections. Resistance to nalidixic acid, doxycycline and tetracycline was also found. The high incidence of plasmid-mediated tetracycline resistance and the emergence of ciprofloxacin resistance in *C. jejuni* poultry isolates supports the need for antimicrobial resistance surveillance.

The presence of the pVir plasmid in the *C. jejuni* isolates was confirmed using two different probes for Southern hybridization. A high incidence of the plasmid

was found in these isolates in comparison to previous studies which have been completed on clinical isolates of *C. jejuni*. Further investigation into why this difference exists is required.

Invasion assays were completed using the Caco-2 cell model and selected *C. jejuni* isolates from both poultry and clinical samples. Results suggested that the Caco-2 cell model may be useful in predicting the virulence of *C. jejuni* isolates.

This thesis also reports work carried out on a collection of erythromycin-resistant *C. jejuni*. A mutation in the 23S rDNA at position 2059 or 2058 (*E. coli* numbering) was found to play a role mediating this resistance. No evidence was generated for the the role of an efflux pump or ribosomal proteins (L22 and L4) in mediating erythromycin resistance in *Campylobacter* species. For one isolate (UA710) no mutation was found in the 23S rDNA suggesting that there may be another mechanism involved in mediating erythromycin resistance.

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Appendix A

Table of Antibiotic Susceptibilities of *Campylobacter jejuni* isolates from Alberta poultry

C. jejuni Isolate No.	Tetracycline R≥16μg/mL[a]	Doxycycline R≥16μg/mL[a]	Ciprofloxacin R≥4μg/mL[a]	Nalidixic Acid R≥32μg/mL[a]
402	64	16	0.023	1.5
407	32	16	0.023	2
412	256	0.016	32	64
416	64	0.064	0.064	3
420	0.032	0.016	0.016	1
426	64	64	0.016	0.75
428	64	16	0.032	1
431	128	32	0.032	0.75
432	128	32	0.023	1
435	64	0.064	0.047	0.75
440	64	16	0.032	1.5
442	64	0.064	0.032	1.5
447	64	64	0.032	1.5
449	256	32	8	8
468	128	64	0.064	8
470	64	64	0.032	1
472	64	32	0.047	0.75

C. jejuni Isolate No.	Tetracycline R≥16μg/mL[a]	Doxycycline R≥16μg/mL[a]	Ciprofloxacin R≥4μg/mL[a]	Nalidixic Acid R≥32μg/mL[a]
479	0.023	0.032	0.016	1.5
481	64	16	0.023	2
483	64	64	0.032	64
490	64	32	0.023	1
494	128	128	0.094	1.5
497	64	32	0.032	1
498	0.023	0.095	0.032	2
509	0.125	0.064	0.094	6
512	0.094	0.094	0.064	2
516	0.047	0.016	0.125	2
521	0.125	0.19	0.125	3
526	32	32	0.032	1
528	32	64	0.023	0.75
529	32	64	0.016	1
533	0.064	0.19	0.047	4
538	64	128	0.125	4
542	0.047	0.047	0.125	6
546	0.064	0.094	0.094	3
548	128	64	0.064	2
557	32	64	0.047	1.5
559	64	64	32	64
561	0.064	0.064	0.094	1.5
566	0.19	0.125	0.38	64
570	128	128	0.047	8
574	128	64	0.25	8
576	32	64	0.032	1.5
578	0.047	0.047	0.023	1
583	64	64	0.032	1
591	32	64	0.023	0.75
592	0.023	0.047	0.047	1.5
598	0.032	0.064	0.032	1
601	64	0.25	32	128
604	64	32	32	256

C. jejuni Isolate No.	Tetracycline R≥16μg/mL[a]	Doxycycline R≥16μg/mL[a]	Ciprofloxacin R≥4μg/mL[a]	Nalidixic Acid R≥32μg/mL[a]
608	32	16	0.125	128
617	128	128	0.064	1.5
619	0.016	0.023	0.125	1.5
625	256	256	0.032	1.5
627	0.016	0.023	0.016	0.38
629	16	0.032	0.094	8
632	64	64	0.125	2
634	64	32	0.012	0.75
638	256	64	0.047	1.5
643	0.047	0.016	0.064	0.75
650	0.125	0.25	0.25	4
653	0.19	0.125	0.032	16
655	512	64	0.032	3
658	0.032	32	0.064	1
661	16	16	0.032	0.75
662	64	4	0.023	1.5
664	64	16	0.032	1
666	128	16	0.032	1.5
669	128	32	0.75	1
672	16	32	0.064	1
674	0.016	0.016	0.094	1
677	32	16	0.094	1
687	0.023	0.016	0.023	0.75
692	0.023	0.023	0.032	1.5
704	0.023	0.023	0.064	1
713	32	16	16	256
714	32	16	16	256
717	128	16	4	256
721	0.023	0.023	0.064	1
724	128	64	0.032	1
730	128	32	0.032	1.5
733	0.023	0.016	0.032	1
735	64	64	0.064	2

C. jejuni Isolate No.	Tetracycline R≥16μg/mL[a]	Doxycycline R≥16μg/mL[a]	Ciprofloxacin R≥4μg/mL[a]	Nalidixic Acid R≥32μg/mL[a]
737	64	16	0.032	0.75
749	64	64	0.094	2
754	16	0.032	0.032	1
755	128	64	0.032	1.5
758	128	32	0.064	1.5
759	0.032	0.064	0.032	1
762	256	64	0.064	2
765	64	16	0.012	0.75
769	64	16	0.032	1
778	64	16	0.016	1.5
780	0.016	0.016	0.016	128
782	64	128	0.125	1.5
783	64	128	0.012	2
788	16	16	0.094	2
789	0.064	0.023	0.032	0.5
798	64	16	0.047	0.75
799	0.016	0.032	0.016	2
802	64	128	0.023	1
804	64	16	0.047	1
805	64	16	0.047	1.5
809	64	32	0.032	1

[a] Susceptible values listed as those obtained by E-test. Resistant values confirmed by agar dilution.